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Sef Is a Spatial Regulator for Ras/MAP Kinase Signaling

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Summary

Spatiotemporal control of the Ras/ERK MAP kinase signaling pathway is among the key mechanisms for regulating a wide variety of cellular processes. In this study, we report that human Sef (hSef), a recently identified inhibitor whose action mechanism has not been fully defined, acts as a molecular switch for ERK signaling by specifically blocking ERK nuclear translocation without inhibiting its activity in the cytoplasm. Thus, hSef binds to activated forms of MEK, inhibits the dissociation of the MEK-ERK complex, and blocks nuclear translocation of activated ERK. Consequently, hSef inhibits phosphorylation and activation of the nuclear ERK substrate Elk-1, while it does not affect phosphorylation of the cytoplasmic ERK substrate RSK2. Downregulation of endogenous hSef by hSef siRNA enhances the stimulus-induced ERK nuclear translocation and the activity of Elk-1. These results thus demonstrate that hSef acts as a spatial regulator for ERK signaling by targeting ERK to the cytoplasm.

Introduction

The Ras/ERK MAP kinase signaling pathway regulates a vast array of cellular responses to extracellular stimuli (Sturgill and Wu, 1991; Nishida and Gotoh, 1993; Robinson and Cobb, 1997; Lewis et al., 1998; Hunter, 2000; Schlessinger, 2000; Chang and Karin, 2001). Extracellular stimuli, such as growth factors, induce sequential activation of three protein kinases, Raf, MEK, and ERK, in the Ras/ERK signaling pathway. MEK always localizes to the cytoplasm by its nuclear export signal (NES) sequence (Fukuda et al., 1996), and cytoplasmic localization of ERK in quiescent cells appears to be achieved by its binding to the N-terminal region of MEK (Fukuda et al., 1997). In response to stimulation, MEK phosphorylation of ERK induces the activation of ERK and its dissociation from the MEK-ERK complex (Adachi et al., 1999). Dissociated, activated ERK then translocates from the cytoplasm to the nucleus (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993; Khokhlatchev et al., 1998), where ERK phosphorylates and activates several nuclear ERK targets, including transcription factors, such as Elk-1 (Treisman, 1996). On the other hand, part of activated ERK remains in the cytoplasm and phosphorylates cytoplasmic ERK targets, such as RSK (Sturgill et al., 1988). Nuclear translocation of ERK is required for growth factor-induced gene expression and reinitiation of cell proliferation (Brunet et al., 1999; Pouyssegur and Lenormand, 2003). Recent reports have shown that, in addition to MEK, phosphoprotein enriched in astrocytes 15 kDa (PEA-15) is also able to act as a cytoplasmic anchor for ERK. PEA-15, which also contains NES, binds both ERK and RSK2 constitutively and thus retains both ERK and RSK2 in the cytoplasm of astrocytes (Formstecher et al., 2001; Vaidyanathan and Ramos, 2003), although how this anchoring is regulated remains to be answered.

A series of recent reports has demonstrated that a significant portion of Ras is localized and activated at the Golgi apparatus and the endoplasmic reticulum (ER) in response to EGF stimulation (Choy et al., 1999; Chiu et al., 2002; Bivona and Philips, 2003; Bivona et al., 2003). Activation of Ras at the Golgi apparatus is dependent on the Src/PLCγ/Ca^{2+}/RasGRP1 signaling pathway (Bivona and Philips, 2003; Bivona et al., 2003), which is distinct from the Grb2/SOS pathway at the plasma membrane. Thus, like activation of Ras on different subcellular compartments, activation of the downstream MEK-ERK pathway may also occur near or at various compartments, including the Golgi and ER.

Recent studies have identified several inhibitors for Ras/ERK signaling, and among them Sprouty and Spred are found to regulate the strength or duration of signaling (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Wakioka et al., 2001; Wong et al., 2002; Hanafusa et al., 2002; Sasaki et al., 2003; Kato et al., 2003). Most recently, Sef (similar expression to *fgf* genes) was identified in zebrafish as an inhibitor of Ras/MAPK-mediated FGF signaling (Tsang et al., 2002; Furthauer et al., 2002). Sef has a putative signal peptide and a putative transmembrane domain and thus is believed to be a transmembrane protein (Tsang et al., 2002; Furthauer et al., 2002; Lin et al., 2002; Kawakami et al., 2003). Sef has been identified in other vertebrates and thus is thought to be a conserved inhibitor of FGF signaling (Tsang et al., 2002; Furthauer et al., 2002; Lin et al., 2002; Niehrs and Meinhardt, 2002; Kawakami et al., 2003; Preger et al., 2004). Vertebrate Sef is expressed in highly restricted patterns in early stages of embryos, and its expression pattern is similar to the expression patterns of *fgf* genes such as *fgf3, fgf8, and fgf17* and *sprouty* members such as *sprouty2* and *sprouty4* (Tsang et al., 2002; Furthauer et al., 2002; Lin et al., 2002; Kawakami et al., 2003). In *Xenopus* embryos, injection of zebrafish Sef (zSef) mRNA was found to inhibit FGF-induced expression of *Xbra*, a panmesodermal marker (Tsang et al., 2002).

There have been, however, contradicting reports concerning the action point of Sef (Furthauer et al., 2002; Kovalenko et al., 2003; Xiong et al., 2003; Yang et al., 2003; Preger et al., 2004). In the first report, zSef was shown to inhibit phosphorylation of ERK induced by either constitutively active Ras or constitutively active MEK, suggesting that Sef acts downstream of or at MEK

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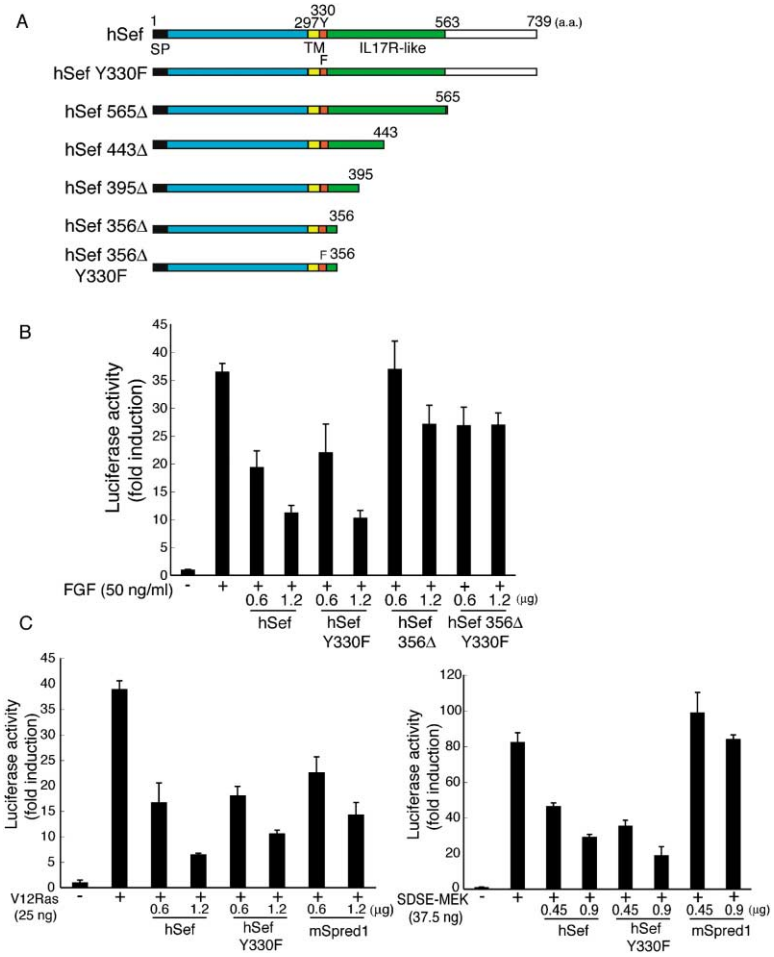


Figure 1. The Action Point of Sef in Ras/ERK Signaling

(A) hSef constructs used in this study. SP, signal peptide (black); putative extracellular domain (blue); TM, putative transmembrane domain (yellow); juxtamembrane domain (red); IL17R-like, similar to IL17 receptor domain (green).

(B) For luciferase assays, HEK293 cells were transfected with pFA-Elk-1, pFR-luciferase plasmids, and increasing amounts of hSef constructs and treated with 50 ng/ml basic FGF before the activity was measured. Error bars indicate SEM (n = 3).

(C) Effect of hSef, hSef Y330F, and mSpred1 on active Ras (V12Ras)- or active MEK (SDSE-MEK)-induced activation of Elk-1.

(Furthauer et al., 2002). In agreement with this, there are reports indicating that human Sef (hSef) is able to reduce phosphorylation of ERK but not phosphorylation of MEK (Yang et al., 2003; Preger et al., 2004). In contrast, other reports argue that Sef inhibits FGF signaling at the level of FGF receptor, as Sef, by binding to FGF receptor, is able to inhibit FGF-induced phosphorylation of FGF receptor and subsequent phosphorylation of FRS2, a substrate of FGF receptor (Kovalenko et al., 2003). Furthermore, Sef was reported to inhibit FGF- or constitutively active FGF receptor-induced phosphorylation of ERK but not active Ras-induced phosphorylation of ERK (Kovalenko et al., 2003; Xiong et al., 2003) or active MEK-induced phosphorylation of ERK (Xiong et al., 2003). Consistent with these observations, there is a report showing that mouse Sef is able to specifically inhibit FGF-induced phosphorylation of ERK without inhibiting PDGF-BB-, EGF-, or calf serum-induced phosphorylation of ERK in NIH3T3 cells (Kovalenko et al., 2003). In contrast, however, overexpression of hSef was reported to inhibit not only FGF-induced differentiation of PC12 cells but also NGF-induced differentiation (Xiong et al., 2003). Thus, molecular mechanisms of actions of Sef have been controversial.

Our detailed analyses here demonstrate that hSef binds to activated MEK specifically, inhibits the dissociation of the MEK-ERK complex, and thus blocks nuclear translocation of activated ERK without inhibiting the activity of ERK in the cytoplasm. Furthermore, our results show that hSef localizes mainly to the Golgi apparatus in unstimulated cells, and after stimulation part of hSef translocates to the plasma membrane region. In stimulated cells, activated MEK and activated ERK colocalize with hSef in both the Golgi apparatus and plasma membrane regions. Then, hSef inhibits the stimulus-dependent phosphorylation of the nuclear ERK substrate Elk-1, whereas hSef does not inhibit phosphorylation of the cytoplasmic ERK substrate RSK2. Remarkably, hSef siRNA experiments have clearly demonstrated that down-regulation of endogenous hSef enhances the stimulus-dependent ERK nuclear translocation and the activation of Elk-1 and thus upregulates the expression of SRE-regulated ERK target genes, such as *c-fos*, *egr1*, and *junB*. All these results demonstrate that hSef is a regulator for intracellular localization of the Ras/ERK pathway and acts as a stimulus-dependent spatial regulator for ERK signaling by blocking signaling to the nucleus and allowing signaling to the cytoplasm.

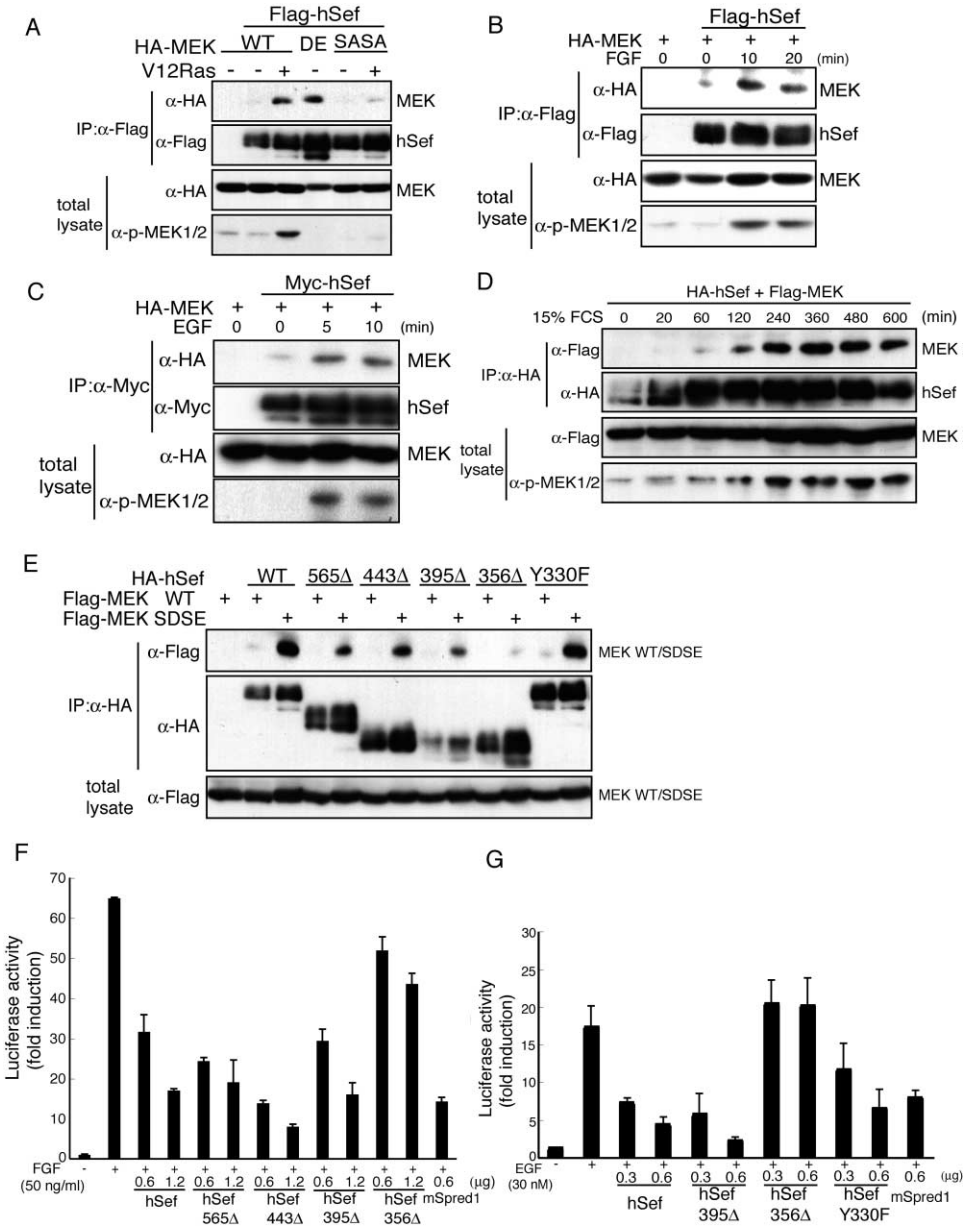


Figure 2. Binding of hSef to Activated Forms of MEK Is Required for the Inhibitor Activity of hSef

(A) C2C12 cells were transfected with Flag-hSef and HA-MEK (wild-type [WT], SDSE-MEK [DE], SASA-MEK [SASA]) with or without V12Ras. HA-MEK constructs that bound to Flag-hSef were detected by immunoprecipitation (IP) with an antibody against Flag followed by immunoblotting with an antibody against HA.

(B and C) hSef binds to phosphorylated MEK in response to FGF (B) or EGF (C) stimulation.

(D) C2C12 cells were transfected with HA-hSef and Flag-MEK and stimulated with 15% fetal bovine serum (FCS) for indicated times.

(E) Binding of hSef constructs to SDSE-MEK. Only hSef 356Δ did not bind to SDSE-MEK.

(F and G) Effect of hSef constructs and mSpred1 on FGF- or EGF-induced activation of Elk-1 in HEK293 cells (F) or HeLa cells (G). Only hSef 356Δ failed to inhibit the activation of Elk-1. Experiments were carried out as in Figure 1B.

Results and Discussion

hSef Inhibits Ras/ERK Signaling Downstream of or at MEK

To examine the action mechanisms of hSef, we constructed various C-terminal deletion mutants and a YF mutant in which a putative regulatory tyrosine residue (Y330, corresponding to Y329 of zSef [Tsang et al., 2002]) is replaced by phenylalanine (Figure 1A). In a reporter assay measuring the transcription activity of Elk-1, a nuclear target of ERK, full-length hSef and hSef Y330F but not hSef 356Δ or hSef 356Δ Y330F, inhibited FGF-induced activation of Elk-1 in a dose-dependent manner (Figure 1B), suggesting that a C-terminal half but not a conserved tyrosine residue is important for the inhibitor activity of hSef. This result is different from the previous

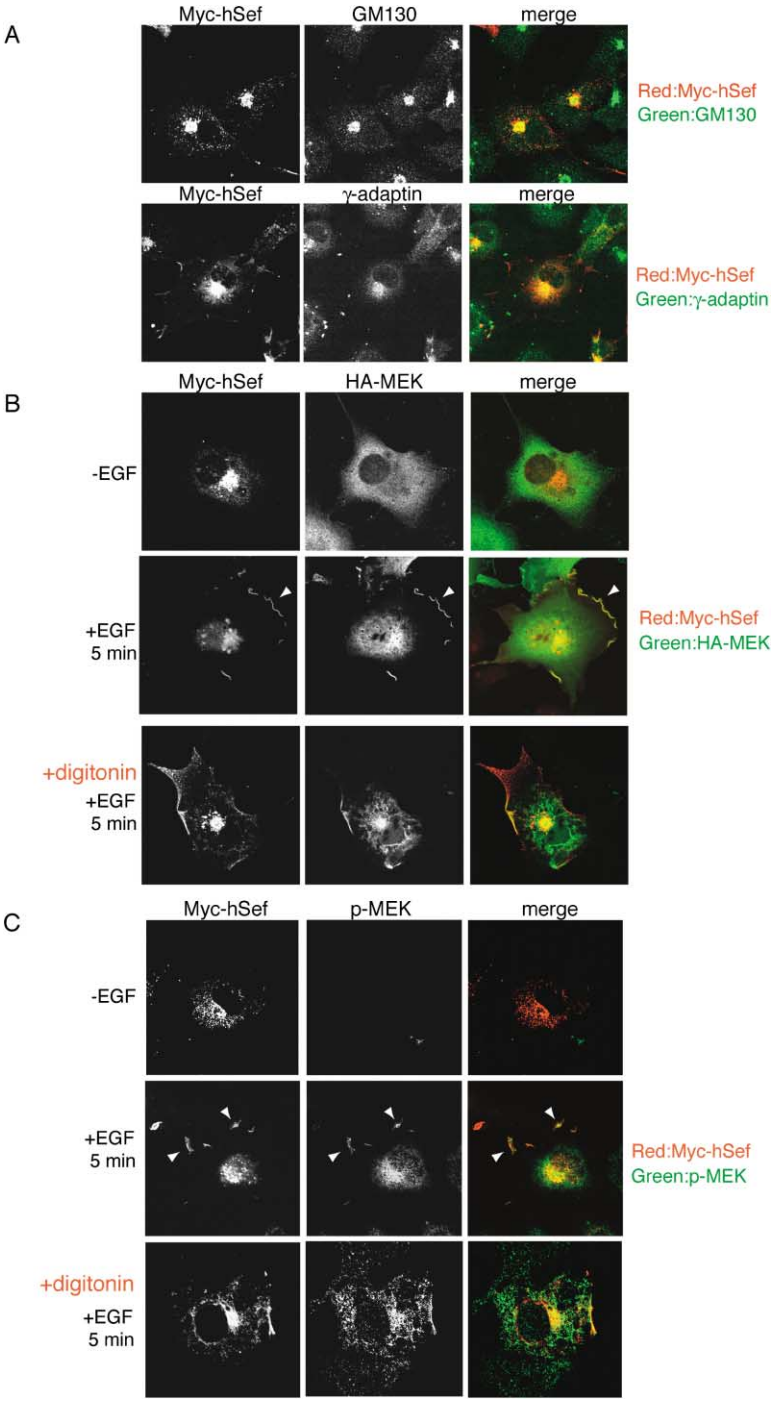


Figure 3. hSef Colocalizes with Activated MEK at the Golgi Apparatus and Ruffling Plasma Membrane Regions

(A) Subcellular distribution of Myc-hSef and GM130 or γ-adaptin in COS7 cells.

(B and C) Subcellular localization of Myc-hSef and HA-MEK (B) or Myc-hSef and phosphorylated MEK (C) in COS7 cells. COS7 cells were transfected with Myc-hSef and HA-MEK and stimulated with EGF for 5 min. Cells of bottommost of each panel are treated with digitonin. Arrowheads indicate ruffling plasma membrane regions.

one for zSef (Tsang et al., 2002), which might result from differences in the assay system and species of Sef that were used. As hSef inhibited FGF-induced activation of Elk-1, we next examined whether expressed hSef inhibits the expression of SRE-regulated ERK target genes, such as *egr1*. In NIH3T3 cells, the percentage of Egr1-positive cells, which was measured by an anti-Egr1 antibody, was increased from 8% in unstimulated cells to 70% in FGF-stimulated cells. When hSef was expressed, the percentage of Egr1-positive cells in hSef-expressing ones was decreased to 32% in FGF-stimulated cells (see Supplemental Figure S1A at http://www.developmentalcell.com/cgi/content/full/7/1/33/DC1). Then, we examined the effect of hSef on the FGF-induced S phase entry of quiescent cells by using the BrdU incorporation assay. When GFP and control vectors were microinjected into serum-starved NIH3T3 cells, the percentage of BrdU-positive cells was increased from 6% in unstimulated cells to 41% in FGF-stimulated (for 16 hr) cells. When GFP and hSef-expressing vectors were microinjected, the percentage of BrdU-positive cells was decreased to 23% in FGF-stimulated

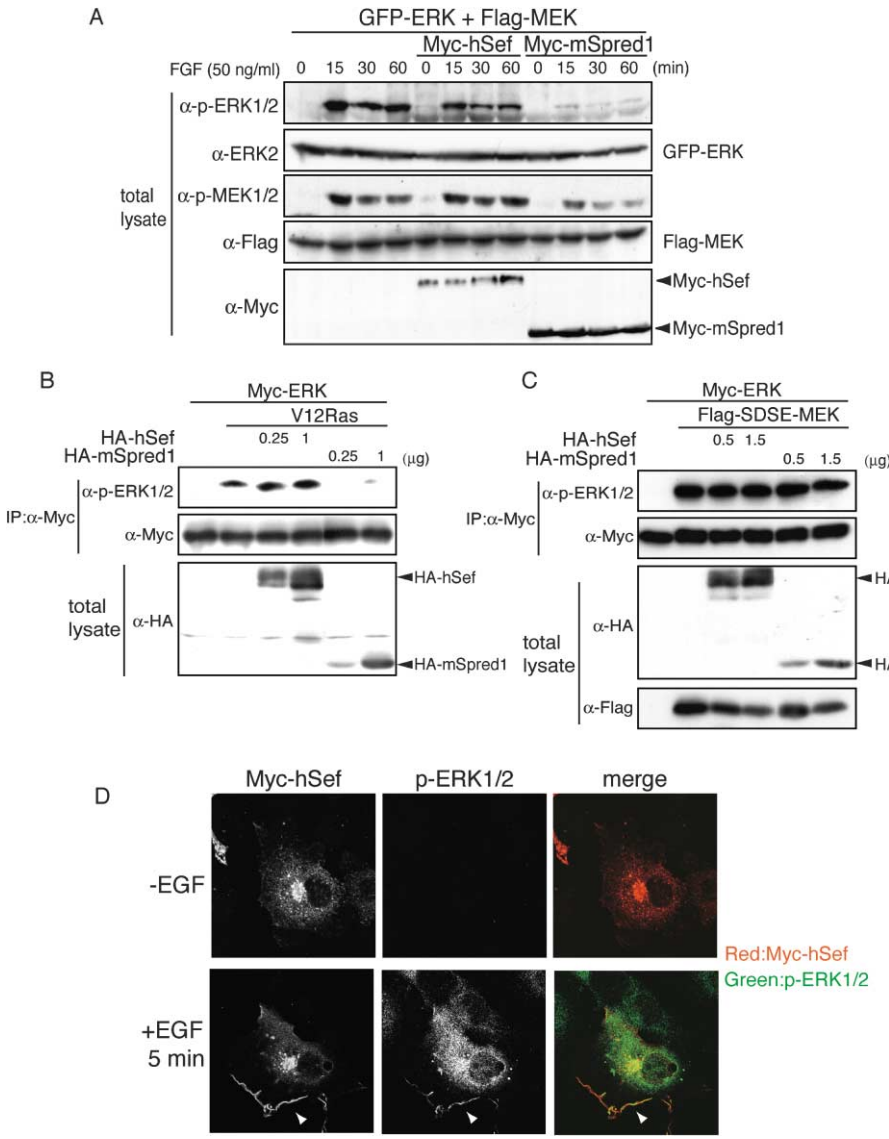


Figure 4. hSef Does Not Inhibit Phosphorylation of ERK

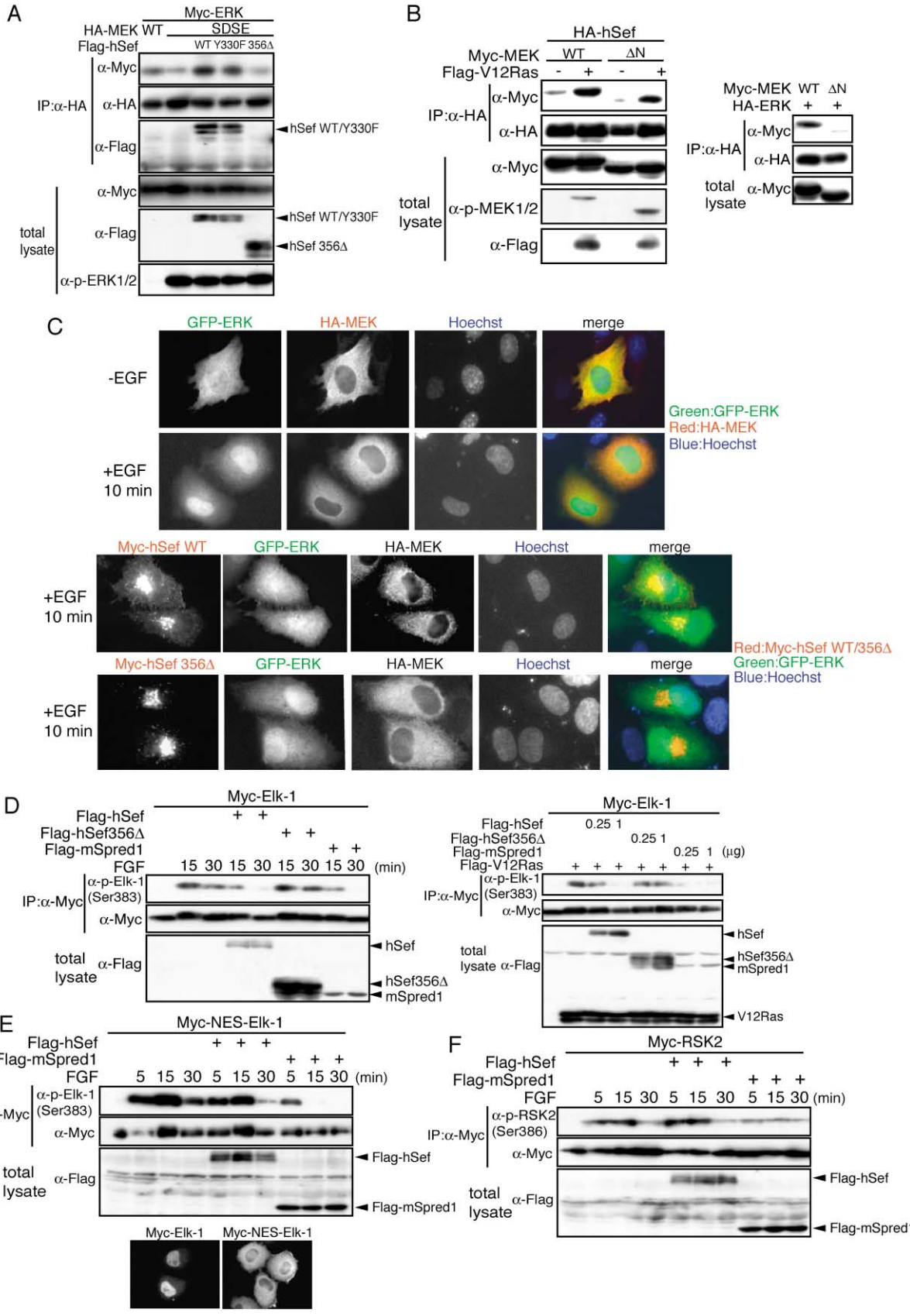
(A) HEK293 cells were transfected with the indicated combinations of cDNAs, stimulated with FGF (50 ng/ml) for the indicated times, and analyzed by immunoblotting with indicated antibodies.

(B and C) C2C12 cells were transfected with the indicated combinations of cDNAs and analyzed by immunoprecipitation with an antibody against Myc followed by immunoblotting with an antibody against phosphoERK1/2.

(D) Colocalization of hSef with phosphorylated ERK1/2 at the Golgi apparatus and ruffling plasma membrane region in response to EGF stimulation. COS7 cells were transfected with Myc-hSef, HA-MEK, and HA-ERK. Arrowheads indicate ruffling plasma membrane regions.

cells (see Supplemental Figure S1A at http://www.developmentalcell.com/cgi/content/full/7/1/33/DC1). Thus, hSef could inhibit expression of ERK target genes and suppress the growth factor-induced cell cycle progression.

There have been contradicting reports concerning the action point of Sef, downstream of MEK (Furthauer et al., 2002; Yang et al., 2003; Preger et al., 2004) or at the level of FGF receptor (Kovalenko et al., 2003; Xiong et al., 2003). To address this, we used mouse Spred1 (mSpred1) as a control, because mSpred1 was found to inhibit Ras/ERK signaling downstream of Ras and upstream of MEK in response to FGF or EGF stimulation (Wakioka et al., 2001; Kato et al., 2003). In agreement with the previous report (Wakioka et al., 2001), mSpred1 inhibited active Ras-induced activation of Elk-1 but not active MEK-induced activation in our reporter assay (Figure 1C). Under the same conditions, hSef inhibited active MEK-induced activation as well as active Ras-induced activation of Elk-1 (Figure 1C), indicating that hSef inhibits Ras/ERK signaling downstream of or at MEK. Consistent with this, hSef is able to inhibit EGF-induced activation of Elk-1 as well as FGF-induced activation (see Figure 2G).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total lysate
α-Flag
Flag-hSef
Flag-mSpred1

Binding of hSef to Activated Forms of MEK Is Required for the Inhibitor Activity of hSef

We considered the possibility that the action point of hSef might be at MEK itself. To test this idea, we examined a possible binding between hSef and MEK. Immunoprecipitation experiments have shown that hSef is able to bind to activated forms of MEK. Under nonstimulating conditions, hSef bound to the constitutively active mutant of MEK (SDSE-MEK, a phosphorylation-mimicking mutant) but not to wild-type MEK (Figure 2A). When active Ras was coexpressed, hSef became bound to MEK (Figure 2A). Even in the presence of active Ras, a nonphosphorylatable (i.e., nonactivatable) mutant of MEK (SASA-MEK) did not bind to hSef significantly (Figure 2A). In addition, when cells were stimulated with FGF, EGF, or 15% fetal bovine serum, hSef bound to MEK in a stimulus-dependent manner (Figures 2B–2D). In all these experiments, the extent of the binding between hSef and MEK correlated very well with the extent of phosphorylation of MEK (Figures 2A–2D). These results taken together indicate that hSef binds to activated MEK.

We then tested various deletion mutants of hSef for their ability to bind to MEK and their inhibitor activity against Elk-1. In the binding assay, wild-type hSef, hSef 565Δ, hSef 443Δ, and hSef 395Δ but not hSef 356Δ bound to active MEK significantly (Figure 2E). Correspondingly, wild-type hSef, hSef 565Δ, hSef 443Δ, and hSef 395Δ but not hSef 356Δ inhibited FGF- or EGF-induced activation of Elk-1 in a dose-dependent manner (Figures 2F and 2G). Thus, there is a very good correlation in these hSef mutants between the ability to bind to activated MEK and the inhibitor activity against Elk-1.

hSef Colocalizes with Activated MEK at the Golgi Apparatus and Plasma Membrane Regions

To confirm the stimulus-dependent interaction of hSef with MEK, we determined subcellular distribution of hSef and MEK. Sef is shown to have a putative signal peptide and a transmembrane domain (Tsang et al., 2002; Furthauer et al., 2002; Lin et al., 2002; Kawakami et al., 2003) and is thus believed to be a transmembrane protein. Biochemical fractionation experiments showed that expressed Sef is present in membrane fractions (see Supplemental Figure S1B at http://www.developmentalcell.com/cgi/content/full/7/1/33/DC1). When expressed in COS7 cells, the majority of hSef localized to the Golgi apparatus, as shown by colocalization with GM130 or γ-adaptin (Figure 3A). As MEK localizes to the cytoplasm diffusely due to its nuclear export signal (Fukuda et al., 1996), hSef and MEK do not colocalize at all under nonstimulating conditions (Figure 3B). After EGF treatment for 5 min, however, part of hSef translocated to the plasma membrane region (Figure 3B), and part of MEK translocated to the same plasma membrane region and to the Golgi apparatus (Figure 3B). Our time-lapse microscopy experiments showed that part of hSef, which localized on membranous vesicles, could move constitutively (data not shown). After EGF stimulation, the part of hSef on vesicles may rapidly translocate to the plasma membrane. The precise mechanism and the mode of this translocation are unknown at present. To confirm more clearly that hSef colocalizes with MEK at the Golgi apparatus, we used digitonin treatment. Treatment with the weak detergent digitonin perforates the plasma membrane, but the nucleus and membranous organelles, such as the Golgi apparatus, remain relatively intact (Adam et al., 1990; Matsubayashi et al., 2001). In digitonin-treated cells, colocalization of hSef and MEK on the Golgi after EGF stimulation was more clearly seen (Figure 3B, bottom). Immunofluorescence with an anti-phosphoMEK antibody showed localization of phosphorylated, activated MEK at the Golgi apparatus and the plasma membrane regions with hSef after EGF stimulation (Figure 3C). Again, in digitonin-treated cells, colocalization of phosphorylated, activated MEK with hSef at the Golgi apparatus was more clearly seen. Recent reports have shown that part of Ras is localized and activated at the Golgi apparatus in response to EGF stimulation (Choy et al., 1999; Chiu et al., 2002; Bivona and Philips, 2003; Bivona et al., 2003). hSef at the Golgi could bind well to MEK, which is activated downstream of Ras at the Golgi. Thus, activated MEK colocalizes with hSef in stimulated cells at both the Golgi and plasma membrane regions.

hSef Does Not Inhibit Phosphorylation of ERK and Colocalizes with Phosphorylated ERK as Well as Phosphorylated MEK

To determine subsequent events after the stimulus-dependent binding of hSef to activated MEK, we then examined whether hSef inhibits phosphorylation and activation of ERK. We thought that hSef would inhibit phosphorylation of ERK, because hSef inhibits strongly the activation of Elk-1, a well-known ERK substrate. Rather surprisingly, however, hSef did not inhibit FGF-stimulated phosphorylation of ERK or MEK in HEK293 cells (Figure 4A). Under the same conditions, mSpred1 strongly inhibited phosphorylation of ERK and MEK (Figure 4A). Essentially the same results were obtained in EGF-stimulated HeLa cells and EGF-stimulated COS7

Figure 5. hSef Inhibits the Dissociation of MEK-ERK Complex and Nuclear Translocation of ERK

(A) hSef WT and hSef Y330F but not hSef 356Δ inhibit the dissociation of the MEK-ERK complex. C2C12 cells were transfected with the indicated combinations of cDNAs and analyzed by immunoprecipitation with an antibody against HA followed by immunoblotting with antibodies against Myc and Flag.

(B) hSef binds to the N-terminal deletion mutant of MEK. C2C12 cells were transfected with HA-hSef and Myc-MEK (WT or ΔN) with or without V12Ras. Myc-MEK constructs that bound to HA-hSef were detected by immunoprecipitation with an antibody against HA followed by immunoblotting with an antibody against Myc (left). MEK ΔN did not bind to ERK (right).

(C) hSef WT but not hSef 356Δ inhibits nuclear translocation of ERK. HeLa cells were transfected with the indicated combinations of cDNAs and stimulated with EGF for 10 min.

(D–F) HeLa cells were transfected with indicated combinations of cDNAs and stimulated with active Ras or FGF for the indicated times. Subcellular localization of Myc-Elk-1 or Myc-NES-Elk-1 is shown.

cells (data not shown). Moreover, while mSpred1 inhibited active Ras-induced phosphorylation of ERK, hSef did not (Figure 4B). In addition, hSef and mSpred1 were unable to inhibit active MEK-induced phosphorylation of ERK (Figure 4C). Correspondingly, hSef did not inhibit the active Ras- or active MEK-induced activation of the kinase activity of ERK (see Supplemental Figure S1C at http://www.developmentalcell.com/cgi/content/full/7/1/33/DC1). Immunofluorescence experiments confirmed that hSef does not inhibit phosphorylation of ERK (Figure 4D). In COS7 cells, where hSef, MEK, and ERK were overexpressed, phosphorylated ERK as well as phosphorylated MEK was detected after EGF treatment for 5 min and colocalized with hSef at the Golgi apparatus and the ruffling plasma membrane regions (Figure 4D; see also Figure 3C). These observations demonstrate that hSef does not inhibit phosphorylation/activation of ERK and suggest the possibility that hSef would retain activated ERK in the cytoplasm to prevent it from entering the nucleus.

hSef Inhibits the Dissociation of the MEK-ERK Complex and Thus Blocks Nuclear Translocation of ERK

To address this possibility, we first examined whether hSef inhibits the stimulus-dependent dissociation of the MEK-ERK complex, because previous studies have shown that MEK acts as a cytoplasmic anchor for ERK under nonstimulating conditions and that, in response to stimulation, MEK phosphorylation of ERK induces the dissociation of the MEK-ERK complex, resulting in nuclear translocation of dissociated, activated ERK (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993; Fukuda et al., 1996, 1997; Adachi et al., 1999). Coimmunoprecipitation assays showed that wild-type hSef and hSef Y330F, which is also active as an inhibitor, inhibited the active MEK-induced dissociation of the MEK-ERK complex, whereas hSef 356Δ, which is inactive as an inhibitor, did not inhibit the dissociation (Figure 5A). They showed further that wild-type hSef and hSef Y330F but not hSef 356Δ bound to the MEK-ERK complex (Figure 5A). None of the hSef constructs inhibited phosphorylation of ERK (Figure 5A) or its kinase activity (see Supplemental Figure S1C at http://www.developmentalcell.com/cgi/content/full/7/1/33/DC1). These results are consistent with the immunofluorescence data shown above. Our domain analysis using an N-terminal deletion mutant of MEK (MEK ΔN) demonstrated that an N-terminal region (residues 1 through 32) of MEK, which is an essential region for binding to ERK (Figure 5B, right; Fukuda et al., 1997), is dispensable for binding to hSef (Figure 5B, left). This is consistent with the idea that hSef, activated MEK, and ERK form a complex.

Then, we examined whether hSef inhibits ERK nuclear translocation. To this end, we used HeLa cells, in which EGF stimulation is able to induce strong nuclear translocation of ERK (Figure 5C, top). Expression of wild-type hSef but not hSef 356Δ almost completely inhibited EGF-stimulated nuclear translocation of ERK (Figure 5C, bottom). Essentially the same results were obtained in EGF-stimulated COS7 cells (data not shown). All these results taken together indicate that hSef binds to activated MEK, inhibits the stimulus-dependent dissociation of the MEK-ERK complex without inhibiting MEK phosphorylation of ERK, and blocks nuclear translocation of activated ERK. If this scenario is correct, hSef would inhibit phosphorylation of ERK substrates in the nucleus without inhibiting phosphorylation of cytoplasmic ERK substrates. Consistent with the observation that hSef but not hSef 356Δ inhibits the stimulus-dependent activation of Elk-1 (see Figures 2F and 2G), hSef but not hSef 356Δ inhibited FGF-, EGF-, or active Ras-stimulated phosphorylation of Elk-1 on serine 383 (Figure 5D; data not shown). In contrast, when Elk-1 was expressed in the cytoplasm by fusing the NES sequence of MEK to Elk-1 (NES-Elk-1), the stimulus-dependent phosphorylation of NES-Elk-1 was not inhibited significantly by hSef (Figure 5E, top). Cytoplasmic localization of NES-Elk-1 was confirmed by immunofluorescence (Figure 5E, bottom). RSK2 is a well-known ERK substrate in the cytoplasm (Sturgill et al., 1988) and has been shown to undergo autophosphorylation on serine 386 after ERK phosphorylation (Dalby et al., 1998). Our data show that hSef did not inhibit FGF-dependent autophosphorylation of RSK2, whereas mSpred1 did (Figure 5F). Thus, hSef inhibits phosphorylation of nuclear ERK substrates and the expression of ERK target genes without inhibiting phosphorylation of cytoplasmic ERK substrates by specifically blocking ERK nuclear translocation.

hSef siRNA Treatment Enhances the Nuclear Translocation of ERK, the Activity of Elk-1, and the Expression of ERK Target Genes in EGF-Stimulated Cells without Altering Phosphorylation of ERK

Finally, we performed hSef siRNA treatment in HeLa-S3 cells. Treatment with either of the two siRNAs (si1 and si2) specifically downregulated endogenous hSef expression, but treatment with a control siRNA (cont.), a si1 reverse sequence, did not (Figure 6A). Both of the Sef siRNA treatments (si1 and si2) enhanced markedly EGF-, active Ras-, or active MEK-induced activation of Elk-1 activity (Figure 6B; data not shown) without altering significantly phosphorylation and activation of ERK (Figure 6C; data not shown). Moreover, the hSef siRNA treatment enhanced markedly EGF-stimulated ERK nuclear translocation (Figure 6D). For example, the siRNA treatment increased the percentage of the cells that showed strong nuclear accumulation of ERK at 10 min after EGF stimulation from 21% (cont.) to 35% (si1) or 32% (si2). Furthermore, the hSef siRNA treatment enhanced significantly the expression level of *c-fos*, *egr1*, and *junB*, which are well-known ERK target genes, after EGF stimulation (Figure 6E). In contrast, the siRNA treatment did not affect EGF-dependent phosphorylation of RSK2 significantly (Figure 6F). These observations further support our idea that hSef acts as a specific inhibitor of ERK signaling to the nucleus by targeting ERK to the cytoplasm.

hSef Acts as a Spatial Regulator for Ras/ERK Signaling by Targeting ERK to the Cytoplasm

Ras/ERK MAP kinase signaling regulates a vast array of cellular responses to extracellular stimuli, such as

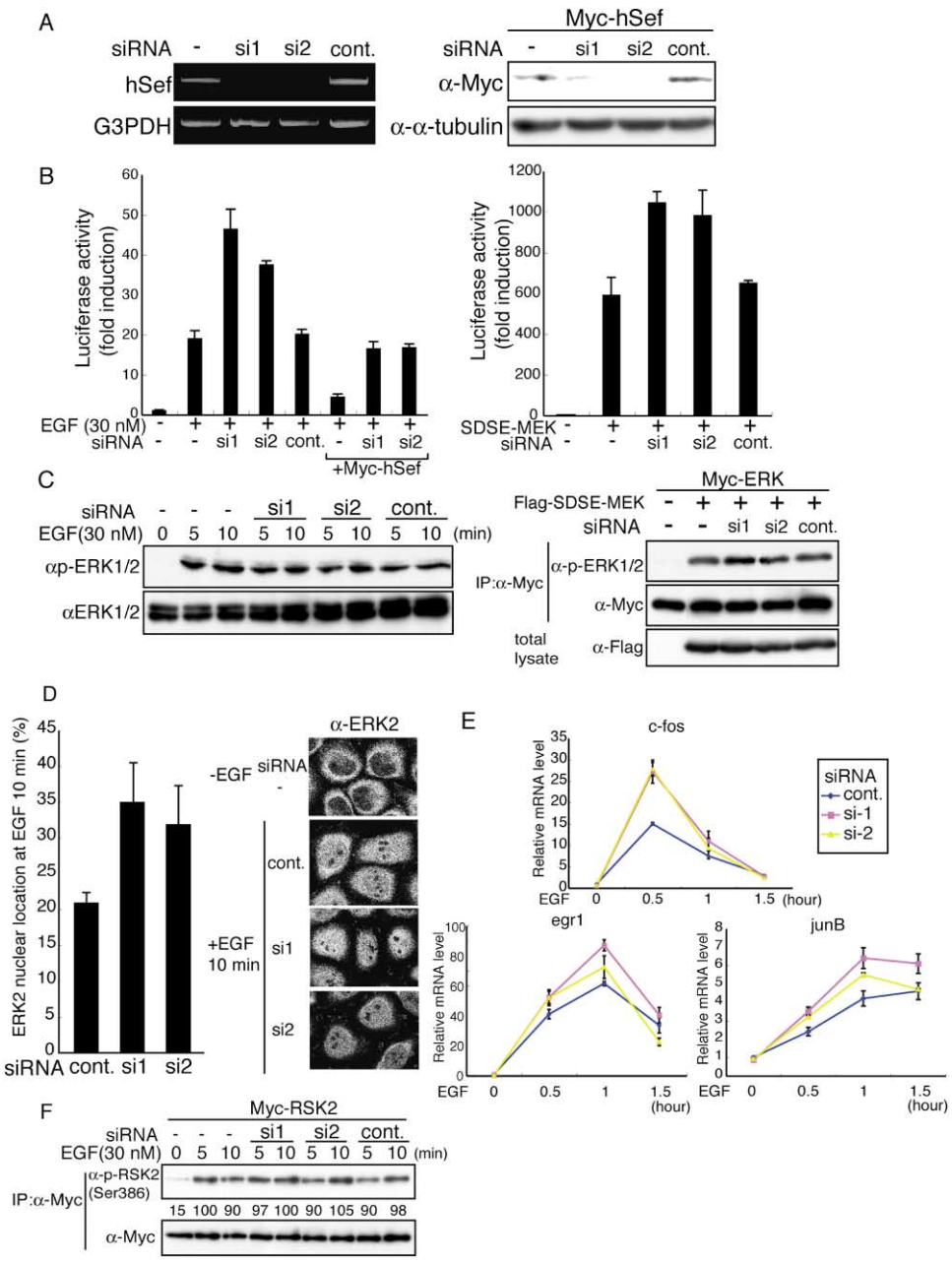

Figure 6. hSef siRNA Treatment Enhances the Nuclear Translocation of ERK, the Activity of Elk-1, and the Expression of ERK Target Genes in EGF-Stimulated Cells without Altering Phosphorylation of ERK

(A) hSef was specifically downregulated in cells by hSef siRNA treatment (si1 or si2). Control siRNA is a reverse sequence of si1. HeLa-S3 cells were treated with either of the hSef siRNAs or a control siRNA, and expression of endogenous hSef was analyzed by RT-PCR using G3PDH as control (left). HeLa-S3 cells expressing Myc-tagged hSef were treated as above, and the cell extracts were prepared and subjected to immunoblotting with antibodies against Myc or α-tubulin as control (right).

(B) hSef siRNA treatment enhances EGF- or active MEK-induced activation of Elk-1 in HeLa-S3 cells. Luciferase activity was measured as in Figure 1B.

(C) hSef siRNA treatment does not affect EGF- or active MEK-induced phosphorylation of ERK.

(D) HeLa-S3 cells were treated with hSef siRNA and incubated in serum-free medium for 24 hr. Then, the cells were treated with or without EGF for 10 min and stained with an antibody against ERK2. More than 200 cells were classified in terms of location of endogenous ERK2, and each percentage of cells in which staining in the nucleus was stronger than that in the cytoplasm is shown (left). Representative images of the cells are shown (right).

(E) hSef siRNA treatment enhances the induction of mRNA of the SRE-regulated genes *c-fos*, *egr1*, and *junB*. HeLa-S3 cells were treated with hSef siRNA or control siRNA. After the cells were incubated in serum-free medium for 24 hr, the cells were incubated with or without EGF (30 nM) for the indicated times. The relative levels of each of the mRNAs were determined by RT-PCR analysis. Each value was normalized to human G3PDH, and the value of control siRNA at EGF treatment for 0 hr of each gene was set to 1. Error bars indicate SEM ($n = 3$). At 0.1 or 1 nM EGF stimulation, the hSef siRNA treatment enhanced the expression of these genes to roughly the same extent (about 1.5- to 2-fold enhancement) as at 30 nM EGF (data not shown).

(F) hSef siRNA treatment does not affect EGF-induced phosphorylation of RSK2. Numbers below the upper panel represent relative intensities of phosphorylation of RSK2. At later time points (at 0.5 or 1.0 hr), no phosphorylation of RSK2 was detected in either control or hSef siRNA-treated cells (data not shown).

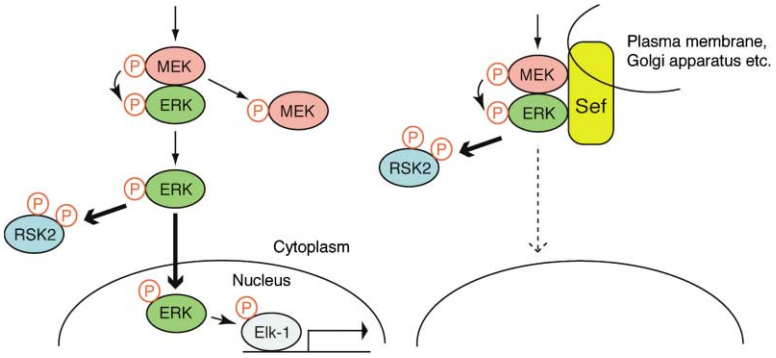

Figure 7. A Model for the Action of Sef

In the absence of Sef, activated MEK phosphorylates ERK, and the phosphorylated ERK dissociates from the MEK-ERK complex. Then, activated ERK phosphorylates RSK2 in the cytoplasm or translocates to the nucleus and phosphorylates Elk-1 (left). Sef binds to activated MEK, inhibits the dissociation of the MEK-ERK complex, and retains activated ERK at the Golgi apparatus or plasma membrane. Thus, Sef specifically inhibits ERK nuclear translocation without inhibiting its activity in the cytoplasm (right).

growth factors (Sturgill and Wu, 1991; Nishida and Gotoh, 1993; Robinson and Cobb, 1997; Lewis et al., 1998; Hunter, 2000; Schlessinger, 2000; Chang and Karin, 2001). It is generally believed that spatiotemporal control of this pathway is a key factor for determining the specificity of cellular responses (Chang and Karin, 2001; Formstecher et al., 2001; Bivona and Philips, 2003; Pouyssegur and Lenormand, 2003; Vaidyanathan and Ramos, 2003). Recently, several inhibitors for Ras/ERK signaling have been identified, and among them Sprouty and related molecules are found to regulate the strength or duration of signaling (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Wakioka et al., 2001; Wong et al., 2002; Hanafusa et al., 2002; Sasaki et al., 2003; Kato et al., 2003). Our results here show that hSef is a regulator for intracellular localization of this pathway and acts to restrict ERK activity to the cytoplasm. Thus, spatiotemporal control of ERK signaling is finely regulated by multiple factors. A remarkable feature of the hSef action is that its interaction with the Ras/ERK signaling pathway is switched on by the activation of this pathway, as hSef specifically binds to activated MEK. Thus, hSef acts as a signaling-dependent regulator for this pathway (Figure 7). The next challenges may include elucidation of molecular mechanisms regulating hSef expression and function.

Experimental Procedures

Molecular Cloning and Plasmid Construction

The full-length cDNA of hSef (GenBank accession number AX350979) was isolated from HEK293 cells by PCR. All hSef constructs were prepared by PCR and ligated into pCS2 containing a Myc, HA, or Flag tag. All tags were added to the C terminus of hSef constructs.

Luciferase Assay

Cells were treated for 15–20 hr with or without 50 ng/ml basic FGF or 30 nM EGF, and the luciferase activity in cell lysates was measured by using the luciferase assay system (Promega, Madison, WI) in a Berthold Lumat LB 9507 luminometer. We normalized the relative luciferase activity to the activity of coexpressed β-galactosidase.

Antibodies, Immunoprecipitation, and Immunoblotting

Antibodies were purchased as follows: antibodies against phosphoERK1/2, phosphoMEK1/2, phosphoElk-1, and phospho-p90RSK from Cell Signaling technology; antibodies against Myc, HA, Egr1, and ERK1/2 from Santa Cruz Biotechnology; antibodies against Flag, γ-adaptin, and α-tubulin from Sigma; an antibody against GM130 from Biosciences; Alexa Fluor 488 goat anti-mouse or rabbit IgG, Alexa Fluor 546 goat anti-rabbit IgG, and Alexa Fluor 594 goat anti-mouse or rabbit IgG from Molecular Probes; Cy5-labeled goat anti-rabbit IgG from Amersham Pharmacia Biotech. Cells were lysed in a buffer comprising 20 mM HEPES (pH 7.5), 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 1% Nonidet P-40, 2 mM dithiothreitol, 1 mM vanadate, 1 mM PMSF, 1% aprotinin, and 10% glycerol. Cell lysates were subjected to immunoprecipitation and immunoblotting with the indicated antibodies.

Cell Cultures and Transfection

C2C12 cells, COS7 cells, HEK293 cells, HeLa-S3 cells, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15%, 10%, 10%, and 10% fetal bovine serum (FBS) and 10% calf serum (CS), respectively. HEK293 cells were cultured on plates coated with collagen. We divided these cells between 35 mm and 60 mm dishes at 1×10^5 and 3×10^5 cells per dish, respectively. After 18 hr, the cells were transfected by using Lipofectamine Plus reagent (Invitrogen) for C2C12 cells and COS7 cells, Lipofectamine 2000 (Invitrogen) for HEK293 cells, and FuGENE6 (Roche) for HeLa cells according to the manufacturer's protocol.

Cell Staining and Digitonin Treatment

Cells were fixed by adding formaldehyde (to a final concentration of 3.7%) directly to the cell culture medium and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. The cells were incubated with indicated primary antibodies at room temperature for 1 hr and then with the appropriate secondary antibodies at room temperature for 1 hr. Cells were finally mounted in Mowiol and examined using a laser scanning confocal microscope (Bio-Rad) or Zeiss Axiophot2. In some cases, after EGF treatment, cells were treated with digitonin (20 μg/ml; Calbiochem) for 5 min on ice. Then, cells were incubated in 4% paraformaldehyde in PBS for 15 min at 37°C and then incubated in methanol for 20 min at −20°C.

siRNA

RNA oligonucleotides (21 nucleotides) homologous to hSef were designed as follows. si1, forward, 5′-GUCGG AGGGA AGACA GUGCT T; reverse, 5′-GCACU GUCUU CCCUC CGACT T. si2, forward, 5′-GCAUG UGAUU GCUGA CGCCT T; reverse, 5′-GGCGU CAGCA AUCAC AUGCT T. Control siRNA, forward, 5′-CGUGA CAGAA GGGAG GCUGT T; reverse, 5′-CAGCC UCCCU UCUGU CACGT T. Cells were treated with annealed siRNAs by the use of Oligofectamine (Invitrogen). By using Alexa Fluor 594 fusion siRNA, we estimated that siRNA transfection efficiency was around 90%.

RT-PCR by Using Light Cycler

Total RNA was reverse transcribed into cDNA by using M-MLV reverse transcriptase (Invitrogen) with oligo random hexamers. Prepared cDNA was purified and subjected to quantitative PCR analysis by using Light Cycler (Roche Diagnostics) with SYBR Green PCR Kit (Qiagen). The primers for the PCR analysis were as follows. For human G3PDH, forward, 5′-TGGGC TACAC TGAGC ACCAG GTGGT; reverse, 5′-CATGT GGGCC ATGAG GTCCA CCAC. For human c-*fos*, forward, 5′-CCAGG GCTGG CGTTG TGAAG; reverse, 5′-CTTGG AGTGT ATCAG TCAGC. For human egr1, forward, 5′-CAGCA CCTTC AACCC TCAGG; reverse, 5′-GTAAC TGGTC TCCAC

CAGCA. For human junB, forward, 5′-CAGCT ACTTT TCTGG TCAGG; reverse, 5′-GTGTA GGCGT CGTCG TGATC.

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