Sef Interacts with TAK1 and Mediates JNK Activation and Apoptosis*

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Sef was recently identified as a negative regulator of fibroblast growth factor (FGF) signaling in a genetic screen of zebrafish and subsequently in mouse and humans. By inhibiting FGFRI tyrosine phosphorylation and/or Ras downstream events, Sef inhibits FGF-mediated ERK activation and cell proliferation as well as PC12 cell differentiation. Here we show that Sef and a dominant negative variant of Sef lacking the extracellular domain (SefIC) physically interact with TAK1 (transforming growth factor-β-associated kinase) and activate JNK through a TAK1-MKK4-JNK pathway. Sef and SefIC overexpression also resulted in apoptotic cell death, while dominant negative forms of MKK4 and TAK1 blocked Sef-mediated JNK activation and attendant 293T cell apoptosis. These investigations reveal a novel activating function of Sef that is distinct from its inhibitory effect on FGF receptor signaling and ERK activation.

Cell growth and differentiation are mediated in part by the actions of receptor tyrosine kinases (RTKs)† that signal via Ras-MAPK pathways (1, 2). Dysregulation of RTK signaling is associated with human diseases including cancer, skeletal dwarfism, and craniosynostosis, thus RTK signaling must be tightly regulated (3–5). One mode involves negative feedback loops that limit the duration and/or intensity of RTK signals.

Within the Ras-MAPK pathway, several feedback inhibitors have recently been identified and include members of the Sprouty and SPRED (Sprouty-related EVH1-domain containing) families of polypeptides (6–8). Sef (similar expression to fgf genes), also called IL-17R-D, is a newly identified feedback inhibitor of fibroblast growth factor receptor signaling and encodes a type I transmembrane domain protein that is highly conserved in zebrafish, mouse, and humans (9). Studies in each system indicate that Sef acts as a feedback inhibitor of FGF-mediated Ras-MAPK signaling and ERK activation (10–13). Studies in mouse also indicate Sef inhibition ofAkt (14). Sef has been proposed to inhibit ERK activation in NIH3T3 cells by physically interacting with the FGFR1 and decreasing its tyrosine phosphorylation (14). Other studies indicate that Sef may act downstream of Ras (12, 15). In addition, a mouse variant of Sef has been identified that gives rise to a cytoplasmic form of Sef that likewise associates with FGFR1 and inhibits ERK activation (15).

In this study, we show that ectopic expression of Sef activates c-Jun amino-terminal kinase (JNK) and apoptosis. We also present evidence that Sef activates JNK through a TAK1-MKK4-JNK pathway and that TAK1 associates with Sef in co-immunoprecipitated complexes. These studies demonstrate for the first time the multifunctional potential of this FGFR and ERK modulating protein as a mediator of JNK and apoptosis.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal antibodies to FLAG and β-actin were from Sigma. The V5 monoclonal antibody was from Invitrogen. Antibodies to JNK, phospho-JNK, c-Jun, phospho-c-Jun, MKK4, phospho-MKK4, caspase-3, and poly(ADP-ribose) polymerase (PARP) were from Cell Signaling and antibodies to His, HA, and TAK1 were from Santa Cruz Biotechnology. SYTOX Green was from Molecular Probes and used according to the manufacturer’s instructions.

Constructions—All Sef constructs were in pcDNA3.1-V5/His and have been described previously (14). Dominant negative MKK4 (dn-MKK4), dominant negative FLAG-tagged TAK1 (dn-TAK1), dominant negative HA-tagged MEKK1 (dn-MEKK1), and dominant negative HA-tagged ASK1 (dn-ASK1) were provide by Dr. Hongbing Shu (National Jewish Medical Center, Denver, CO).

Cell Transfection, Immunoprecipitation, and Immunoblotting—293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfection experiments were carried out using the Genejuicer™ reagent (Novagen) as described previously (14). Cells were harvested 24–36 h after transfection by lysis in HNTG buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1.0 mM EDTA, 0.1 mM NaVO₄). Lysates were either subjected directly to immunoblot analysis or immunoprecipitated overnight with the indicated antibodies. Immune complexes were recovered with protein-A/G-agarose (Santa Cruz Biotechnology), washed in HNTG buffer, and subjected to immunoblotting procedures as described previously (14).

Cell Death Assays—293T cells were transiently transfected with mSef expression plasmids and at 36 h were stained with 1 μM SYTOX Green dye and washed with phosphate-buffered saline. Apoptosis was quantified by calculating the percentage of fluorescence-positive (dead) cells as determined by flow cytometry. Fluorescence positive cells were also visualized by fluorescence microscopy (Olympus IX70), and fluorescent digital images were merged with digitized phase contrast images.

RESULTS AND DISCUSSION

Overexpression studies of full-length mSef and Sef subdomains revealed that mSef and mSefIC (intracellular and transmembrane domain), but not mSefECTM (extracellular and
transmembrane domain), induced apoptotic cell death in transfected 293T cells (Fig. 1, a and b). SYTOX Green staining, a measure of cell death, increased almost 6-fold in 293T cells overexpressing Sef or SefIC but not SefECTM. Similar results were obtained by AnnexinV staining of Sef-transfected cells (data not shown). In part, this result may be partially explained by our previous observation that mSef inhibits FGF-induced Akt phosphorylation, and Akt phosphorylation is known to inhibit apoptosis and promote cell survival (14). To further investigate the mechanisms of mSef-mediated cell death in 293T cells, PARP cleavage was assayed. PARP is a nuclear DNA repair protein that is cleaved by caspase into two subunits rendering it inactive (16, 17). Immunoblot analysis of cells transfected with mSef or mSefIC displayed elevated levels of cleaved PARP, whereas cells transfected with mSefECTM or vector control did not (Fig. 1c). Immunoblotting experiments also revealed that overexpression of Sef and SefIC resulted in caspase-3 activation (Fig. 1d), that parallel PARP cleavage in that activated caspase-3 has been shown to mediate PARP cleavage (16, 17). These data are consistent with the above SYTOX Green staining results (Fig. 1, a and b) and indicate that ectopic expression of mSef and mSefIC results in apoptotic cell death. Data also indicate that this pro-apoptotic activity likely is mediated by the Sef cytoplasmic domain.

Studies in several systems indicate that JNK plays an essential role in apoptosis and this may be mediated by phosphorylation of c-Jun, as well as other targets (18, 19). To determine whether JNK activation is involved in mSef-induced cell death of 293T cells we performed immunoblot analysis of cell lysates from mSef, mSefIC, and mSefECTM-transfected 293T cells using phospho-JNK and phospho-c-Jun specific antibodies (Fig. 2). JNK and c-Jun, one of its immediate downstream targets, were phosphorylated in 293T cells transfected with mSef and mSefIC but not by mSefECTM or vector controls (Fig. 2, a–c). These data suggest a role for JNK and c-Jun phosphorylation in mSef-mediated apoptosis.

To determine whether JNK phosphorylation by ectopic expression of mSef or mSefIC was unique to 293T cells, we also injected Xenopus embryos with mRNAs encoding mSef, mSefIC, or mSefECTM (Fig. 2d). Immunoblot analyses of lysates from gastrula stage embryos overexpressing mSef, mSefIC, or mSefECTM consistently demonstrated that mSef and mSefIC, but not mSefECTM, induce JNK phosphorylation in Xenopus embryos in a manner similar to that of 293T cells. These data indicate that JNK activation by ectopically expressed mSef or mSefIC is not unique to 293T cells.

JNK is activated by multiple signaling pathways including MAP kinase kinase kinases ASK, MEKK1, and TAK1 (19). These in turn activate MKK4 and/or MKK7 to mediate JNK activation. In addition, Rac1, RhoA, and Cdc42 GTPases modulate JNK activity. Therefore, we employed dominant negative forms of Rac1, RhoA, and Cdc42 to determine whether any of these effector proteins might act as intermediates in Sef-mediated JNK activation. Co-transfection of 293T cells with mSef, mSefIC, or mSefECTM consistently demonstrated that mSef and mSefIC, but not mSefECTM, induce JNK phosphorylation in Xenopus embryos in a manner similar to that of 293T cells. These data indicate that JNK activation by ectopically expressed mSef or mSefIC is not unique to 293T cells.

FIG. 1. mSef and mSefIC induce 293T cell apoptosis. HEK293T cells were transfected with mSef (1 μg) constructs for 36 h. a, cells were stained with SYTOX Green dye for 5 min and visualized by phase contrast and fluorescence microscopy. Shown are the merged images. b, SYTOX Green stained cells were quantified by flow cytometry. c, transfected cells were lysed in HNTG buffer and subjected to immunoblot analysis using PARP antibody. Dots were rewarped with anti-β-actin antibodies as a loading control and anti-V5 antibodies to demonstrate Sef expression.

FIG. 2. mSef and mSefIC mediate JNK activation and subsequent c-Jun phosphorylation. a, 293T cells were transfected for 36 h with mSef constructs (1 μg) as indicated. Transfected cell lysates were prepared and subjected to immunoblotting using antibodies against phospho-JNK, JNK, phospho-c-Jun, and c-Jun. The expression of mSef was confirmed by immunoblotting with V5 antibodies. b and c, the fold induction of JNK and c-Jun phosphorylation was calculated as described under “Experimental Procedures” and is representative of three independent experiments. d, Xenopus embryos were injected in the marginal zone at the two-cell stage with mSef, mSefIC, and mSefECTM cRNA and harvested at stage 12. Embryo lysates were analyzed by immunoblotting using phospho-JNK, JNK, and V5 antibodies. β-Gal, β-galactosidase.
Fig. 3. dn-MKK4 and dn-TAK1 inhibit mSef-induced JNK activation and subsequent c-Jun phosphorylation. a, 293T cells were transfected with 1 μg mSef and 0, 0.5, 1.0, 1.5, and 2.0 μg of dn-MKK4 for 36 h. Cell lysates were analyzed by immunoblotting using phospho-JNK, JNK, phospho-c-Jun, c-Jun, HA, and V5 antibodies. b, transfected cells were stained with SYTOX Green dye, and apoptotic cells were quantified by flow cytometry and are representative of three independent experiments. c, 293T cells were transfected with mSef constructs as indicated, and cell lysates were obtained and analyzed by immunoblotting using phospho-MKK4 and MKK4 antibodies. Blots were reprobed, demonstrating Sef expression. d, 293T cells were transfected with 1.0 μg of mSef expression plasmid alone or with 1.0 μg of dn-TAK1-FLAG, dn-ASK1-HA, or dn-MEKK1-HA for 24 h. Cell lysates were subjected to immunoblotting with antibodies to phospho-JNK, JNK, phospho-c-Jun, FLAG (dn-TAK1), or HA (dn-ASK1 and dn-MEKK1). Immunoblots were visualized by enhanced chemiluminescence.

decreases in SYTOX Green staining in a dose-response manner consistent with the notion that inhibition of mSef-mediated JNK activation rescues cells, at least partially, from apoptosis (Fig. 3b). The lack of a full rescue from apoptosis by overexpression of dn-MKK4 suggests that there may be additional pathways activated by mSef that also induce apoptosis. Alternatively, the relative stoichiometry of dn-MKK4 to mSef may be unfavorable for a complete rescue from the apoptotic effect. To confirm that MKK4 is indeed involved in this pathway we also examined whether MKK4 was phosphorylated in response to forced expression of Sef, SefIC, or Sef ECTM. Immunoblot analysis indicates that endogenous MKK4 is phosphorylated by overexpression of Sef or SefIC but not by SefECTM (Fig. 3c). These data are consistent with MKK4 acting downstream of Sef and upstream of JNK.

Since several kinases act as upstream activators of MKK4 we tested dominant negative forms of several of these including ASK1, MEKK1, and TAK1 (18, 19). dn-TAK1, but not dn-ASK1 or dn-MEKK1, inhibited mSef-mediated JNK and c-Jun phosphorylation (Fig. 3d). SYTOX Green staining of 293T cells co-transfected with mSef and increasing amounts of dn-TAK1 revealed that dn-TAK1 partially rescues cells from mSef-mediated apoptosis (data not shown). Like dn-MKK4, dn-TAK1-mediated rescue from apoptosis is incomplete, and this may reflect activation of additional apoptotic pathways by overexpression of Sef or SefIC. Taken together, these results are consistent with Sef-mediating apoptosis via a TAK1-MKK4-JNK pathway. TAK1 has also been implicated in activation of the p38 MAPK pathway (19). Immunoblot analysis of 293T cell lysates transfected with Sef or SefIC with phospho-p38 antibodies showed no increase in p38 phosphorylation above control empty vector transfected cell lysates (data not shown). This indicates that ectopic expression of Sef activates MKK4 via TAK1 resulting in JNK but not p38 activation.

Since the mechanism by which mSef mediates JNK activation and apoptosis is not known, we sought to determine whether Sef associates with any of the components of the TAK1-MKK4-JNK pathway. Co-transfection and immunoprecipitation experiments revealed that TAK1 associates with mSef and mSefIC but not mSefECTM, and this is consistent with the role of the cytoplasmic domain of mSef as a mediator of JNK activation and apoptosis (Fig. 4, a and b). To confirm this interaction, immunoprecipitation of cell lysates from mSef, mSefIC, or mSefECTM transfected cells with anti-TAK1 antibodies were performed and revealed that mSef and mSefIC also interact with endogenous TAK1 (Fig. 4c).

Overall, the present studies suggest that Sef is a multifunctional protein whose first function was identified as that of an inhibitor of FGF signaling (9–12, 14, 20). Several studies show that Sef inhibits FGF-induced ERK activation, although the mechanism by which this occurs remains in doubt (9). Previously, we have shown that Sef inhibits FGF-mediated activation of FGRFR1 that inhibits all downstream signaling from the receptor in NIH3T3 cells (14). Another study shows that Sef inhibits FGF-induced PC12 cell differentiation upstream of Ras (20), presumably by an interaction with FGRFR1. However, an alternate mechanism has been proposed by evidence that suggests that Sef acts downstream of Ras at the level of MEK (10, 12). In the present study we show that mSef when overexpressed in 293T cells induces JNK activation and apoptosis, indicating that mSef has a function in addition to inhibiting FGF-mediated ERK activation (9). Our data show that Sef-
mediated apoptosis occurs, at least in part, via a Sef-TAK1-MKK4-JNK pathway. The mechanism by which mSef activates TAK1 remains to be determined, although recently hSef has been shown to form homodimers (10), and this might promote association of Sef with TAK1. Furthermore, while we have demonstrated that mSef co-immunoprecipitates with epitope-tagged TAK1 and endogenous TAK1, we cannot exclude the possibility that an additional protein or proteins may be required for the formation or stabilization of this complex. Additional studies will elucidate the mechanism by which mSef induces JNK phosphorylation and apoptosis and the conditions required for this activation.

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