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Sphingosine Kinase 1 is a Critical Component of the Copper-Dependent FGF1 Export Pathway

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Abstract

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This article is dedicated to the memory of Tom Maciag, scientist and friend.

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Sphingosine kinase 1 catalyzes the formation of sphingosine-1-phosphate, a lipid mediator involved in the regulation of angiogenesis. Sphingosine kinase 1 is constitutively released from cells, even though it lacks a classical signal peptide sequence. Because copper-dependent non-classical stress-induced release of FGF1 also regulates angiogenesis, we questioned whether sphingosine kinase 1 is involved in the FGF1 release pathway. We report that (i) the coexpression of sphingosine kinase 1 with FGF1 inhibited the release of sphingosine kinase 1 at 37°C; (ii) sphingosine kinase 1 was released at 42°C in complex with FGF1; (iii) sphingosine kinase 1 null cells failed to release FGF1 at stress; (iv) sphingosine kinase 1 is a high affinity copper-binding protein which formed a complex with FGF1 in a cell-free system, and (v) sphingosine kinase 1 over expression rescued the release of FGF1 from inhibition by the copper chelator, tetrathiomolybdate. We propose that sphingosine kinase 1 is a component of the copper-dependent FGF1 release pathway.

Keywords

Sphingosine Kinase 1; Fibroblast Growth Factor 1; Copper; Non-classical Release; Angiogenesis

Introduction

Sphingosine-1-phosphate (S1P), a lipid mediator produced by sphingosine kinase 1 (SK1), is implicated in a variety of biological processes [1,2]. Although high levels of intracellular S1P induce calcium mobilization and enhance cell proliferation and survival [3–5], extracellular S1P acts through specific G-protein-coupled receptors to promote cytoskeletal rearrangement, cell migration, vascular maturation, and angiogenesis [6–11]. It is suggested that these activities are regulated by a dynamic balance between the levels of the sphingolipid metabolites, ceramide and S1P, described as the “sphingolipid rheostat” [12].

SK1 is both a cytosolic and membrane-associated enzyme. It is activated by several biological regulators including tumor necrosis factor- α [13], platelet-derived growth factor [14], nerve growth factor [1,15], muscarinic acetylcholine receptor agonist [16], serum [14], and phorbol esters [17]. The expression of SK1 promotes the G₁-S transition in NIH 3T3 cells, and protects these cells against apoptosis induced by serum deprivation [18]. In addition, it is suggested that SK1 may act as an oncoprotein [19].

Interestingly, SK1 is exported from human umbilical vein endothelial cells [20], SK1-transfected human embryonic kidney 293 cells, and lung smooth muscle cells [21] through a non-classical pathway of release [20]. A number of other extracellular proteins follow various non-classical export routes [22–24]. Among them are potent pro-angiogenic and pro-inflammatory polypeptides such as FGF1 [25], FGF2 [26–28], IL1 α [29], and IL1 β [30,31]. The release of SK1 shares several similarities with FGF1, which is also an important regulator of cell proliferation and migration [32]. Indeed, like FGF1, SK1 lacks a classical signal peptide sequence required for release through the endoplasmic reticulum and Golgi apparatus [20, 25], and its export into the extracellular compartment is brefeldin A-insensitive [20,25], ATP-dependent [20,33], and requires an intact actin cytoskeleton [20,34]. FGF1 is released as a copper-dependent multiprotein complex which includes the EF hand-containing protein S100A13 and the p40 form of the membrane docking protein synaptotagmin 1 (Syt1) [22]. Interestingly, members of the FGF1 release complex are copper-binding proteins [35]. We demonstrated the ability of FGF1 to form high molecular weight aggregates with both S100A13 and p40 Syt1 in the presence of exogenous copper in a cell-free system [35]. Moreover, we reported that the copper chelator tetrathiomolybdate (TM) inhibits the temperature-dependent release of FGF1 [36]. Because (i) FGF1 is exported in response to temperature stress [25,37], (ii) SK1 is implicated in the heat shock response by its ability to enhance cell survival upon severe heat stress in yeast [38,39], and (iii) the export of SK1 [20] exhibits similar

pharmacologic properties to those described for FGF1 release [34,36], we sought to determine whether SK1 is a component of the FGF1 release pathway. We report that SK1 was released from NIH 3T3 cells in response to cellular stress as a component of the FGF1 release complex, and that SK1 knockout resulted in the blockage of FGF1 release. Our data also suggest that SK1 may act as a copper donor facilitating the formation of the FGF1-containing multiprotein release complex.

Materials and Methods

Cell Culture

Murine NIH 3T3 cells (ATCC, Manassas, VA) and stable FGF1 NIH 3T3 [25] cell transfectants were maintained in DMEM (HyClone, Logan, UT) supplemented with 10% bovine calf serum (HyClone, Logan, UT) on human fibronectin-coated dishes (10 $\mu\text{g}/\text{cm}^2$) [25]. Stable transfectants were also supplemented with 0.4 g/l Geneticin (Invitrogen-GIBCO, Carlsbad, CA.). SK1 $+/+$ mouse embryo fibroblast cells and SK1 null mouse embryo fibroblast cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT) on human fibronectin-coated dishes.

Preparation of Adenoviral Constructs and Cell Infection

The SK1 construct cloned into the V5-His-pcDNA3.1 eukaryotic expression vector (Invitrogen, Carlsbad, CA) was excised from the plasmid by digestion with HindIII and PmeI (New England Biolab, Ipswich, MA); and the fragment was subcloned in the multiple cloning site of the adenovirus shuttle vector, pAdlox, using HindIII and SmaI restriction sites. The resulting plasmid was digested with SfiI, resolved by 1% agarose gel electrophoresis, excised, and purified using the QIAquick Gel Extraction kit (Quiagen, Valencia, CA). The fragment containing the SK1 cDNA was cotransfected with Ψ 5 helper virus DNA into mouse epithelial kidney CRE 8 cells; and the recombinant viral product was purified by two sequential CsCl gradient centrifugations as previously described [40]. The production of the adenovirus expressing FGF1 was reported earlier [41]. Additionally, FGF1:HA (gift of Andrew Baird, Institute for Molecular Medicine, La Jolla) was cloned into the EcoRI restriction site of the pAdlox construct, and the virus was produced as described for SK1:V5. NIH 3T3 cells were infected with adenoviruses, as previously described [41].

SK1 Knockout Mouse Embryo Fibroblasts Immortalization

SK1 knockout mouse embryo fibroblasts provided by Dr. R. Proia (NIH, Bethesda, MD), were immortalized by stable transfection with SV40 T-antigen in the psg65 vector (gift of Dr. James DeCaprio, Harvard University) in parallel with normal mouse embryo fibroblasts. The resulting colonies were tested for lack of SK1 expression by RT PCR analysis using specific SK1 primers, as described [42].

Heat Shock and Processing of Conditioned Media

Heat shock-induced non-classical protein release was studied as previously described [34, 36]. NIH 3T3 cell transfectants were grown to 70% confluency (7×10^6 cells per 15 cm Petri dish), and prior to heat shock, the culture medium was changed to DMEM containing 5 U/ml of heparin (Sigma-Aldrich, St. Louis, MO). Following temperature stress, the conditioned media were collected, filtered, treated with 0.1% DTT (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C, and adsorbed to a 1 ml heparin-Sepharose CL-6B column (GE Healthcare, Piscataway, NJ) pre-equilibrated with 50 mM Tris pH 7.4 containing 10 mM EDTA (TEB). The adsorbed proteins were washed with TEB, eluted with TEB containing 1.5 M NaCl, and concentrated (Amicon Centricon 10; Millipore, Billerica, MA). The samples were resolved by 15% SDS-PAGE and immunoblotted with a rabbit polyclonal anti-FGF1 antibody [43]. The

flow through media from the heparin-Sepharose CL-6B column were collected, concentrated using the Ultrafree-15 Centrifugal filter device (Millipore, Billerica, MA), and incubated with a monoclonal anti-V5 antibody (Invitrogen, Carlsbad, CA) overnight at 4°C. Protein G-Sepharose (GE Healthcare, Piscataway, NJ) was added, and the mixture was incubated for 2 hours at 4°C. Immunoprecipitates were resolved by 12% SDS-PAGE and immunoblotted with the monoclonal anti-V5 antibody. Total cell lysates were obtained from the individual populations of cells, as previously described [34]. The loading of cell lysates and conditioned media on electrophoretic gels was standard in all experiments. Each experimental point corresponded to one 15 cm Petri dish of cell culture (7×10^6 cells). Total processed conditioned medium or 1/10 of cell lysate were loaded on one electrophoretic lane. The activity of lactate dehydrogenase (Sigma-Aldrich, St. Louis, MO) in conditioned media was utilized as an assessment of cell lysis in all experiments, as previously described [44]. Densitometric analysis of the Western blots was performed utilizing a phosphorimager (Molecular Dynamic, Pegasus Scientific, Frederick, MD).

Coimmunoprecipitation Experiments

20 ml of conditioned media from 7×10^6 heat shocked or control NIH 3T3 cells adenovirally transduced with FGF1:HA and/or SK1:V5 constructs, were collected, filtered, and concentrated to the volume of 1 ml by using Ultrafree-15 centrifugal filter device (Millipore, Billerica, MA) at 2000 g for 1 hour at 4°C. Similarly, the unbound samples after heparin-Sepharose adsorption were collected and concentrated to the volume of 1 ml. To prepare the cell lysates, the cells were washed twice with PBS and lysed in NPB buffer (20 mM Tris HCl, pH 7.5, containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% glycerol, 2 mM EDTA, 1% Triton X-100, 1 µg/ml leupeptin, and 0.2% deoxycholate). In some experiments, the cells were hypotonically lysed in 5 mM Tris HCl pH 7.4, containing protease inhibitors (50 µg/ml pepstatin, 50 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF), using Dounce homogenization. The lysates were centrifuged at 10,000 g for 15 minutes at 4°C, and the protein concentration was measured by the BCA method (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of lysates and conditioned media were precleared by incubation for 1 hour at 4°C with protein G-Sepharose (GE Healthcare, Piscataway, NJ) and mouse IgG (1 µg/ml). After centrifugation at 10,000 g for 10 minutes, the supernatants were immunoprecipitated with monoclonal anti-HA (Covance Innovative Antibodies) overnight at 4°C. The immunoprecipitates were recovered using protein G-Sepharose affinity, washed four times with NPB buffer, the pellets were solubilized in 60 µl of boiling Laemmli buffer, resolved by 12% SDS-PAGE, and analyzed using anti-V5 and anti-HA antibodies.

Copper Affinity of SK1 and Gel Shift Analysis

In order to evaluate the copper affinity of SK1, it was necessary to delete the His tag sequence from the original SK1 construct. This was achieved by the introduction of a termination codon upstream at nucleotide 1054, which is located at 5' of the His sequence within the V5-His epitope tag. The deletion of the His tag and conservation of the V5 tag was confirmed by the immunofluorescence microscopy of transfected cells using rabbit anti-His antibodies (Santa Cruz, California) and mouse monoclonal anti-V5 antibodies. The mutated SK1:V5-pcDNA3.1 expression plasmid was translated *in vitro* using the T7 Quick *in vitro* transcription and translation system (Promega). The reaction product was adsorbed to a HiTrap chelating column (GE Healthcare, Piscataway, NJ) which was preloaded with 0.1 M CuCl_2 in 20 mM sodium phosphate buffer, pH 7.2, containing 1 M NaCl. The column was eluted with increasing concentrations of imidazole (Sigma-Aldrich, St. Louis, MO) in the equilibration buffer. The eluted fractions were concentrated by using Centricon 10 (Amicon, Millipore, Billerica, MA), resolved by 10% SDS-PAGE, and subjected to V5 immunoblot analysis.

To analyze the interactions between the recombinant forms of SK1 [45] and FGF1 [35,46, 47], the recombinant proteins were mixed at a molar ratio of 1:1, as previously described [35], lyophilized, and resuspended in 50 μ l of phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), in the presence or absence of 1 mM CuCl_2 . The samples were incubated at 42°C for 30 minutes, resuspended in non-reducing SDS-PAGE loading buffer, and resolved in nonreducing and limited denaturing SDS-PAGE gel containing 0.1% SDS as previously described [36]. The samples were then immunoblotted using polyclonal antibodies against FGF1 and SK1. To evaluate the role of SK1 as copper donor, recombinant SK1 was incubated with 500 mM imidazole or 500 μ M TM for 30 minutes at 37°C, dialyzed against PBS, and incubated in a buffer containing FGF1 in the absence of CuCl_2 , as described above. The samples were resuspended in a non-reducing SDS-PAGE loading buffer, and resolved by non-reducing and limited denaturing SDS-PAGE. To verify the specificity of SK1:FGF1 interaction, we used the recombinant C-terminal portion of osteopontin (OPN) as a control (gift of Dr. L. Liaw, MMCRI, Scarborough, ME) in the cell-free system. Increased amounts of SK1 were mixed with 1 μ g of either FGF1 or C-terminal OPN fragment in copper-free conditions and processed as described above. The samples were resolved by non-reducing, limited denaturing SDS-PAGE, followed by the immunoblotting with rabbit antibodies against FGF1 or against OPN.

Results

FGF1 represses the constitutive release of SK1 from NIH 3T3 cells

We previously demonstrated that the coexpression of FGF1 with the small calcium-binding protein, S100A13, which also lacks a signal peptide, results in the blockage of S100A13 release at 37°C but not at 42°C. These data were the first indication that S100A13 is a member of the FGF1 release complex [35]. Thus, we used a similar coexpression approach to evaluate the possibility of SK1 participation in the FGF1 release pathway. Initially, FGF1 release was studied in NIH 3T3 cells [25], therefore we verified the release of SK1 from these cells. Because SK1 is released from human endothelial cells and from SK1-transfected 293 cells *in vitro* at 37°C, and its export is enhanced at 42°C [20], we investigated whether the release of SK1 is heat shock-dependent in NIH 3T3 cells.

The spectrophotometric measurement of extracellular lactate dehydrogenase (LDH) activity [44] is a standard method used in our laboratory to monitor cell damage at stress [25]. The LDH activity values for medium conditioned by NIH 3T3 cells at 37°C vary between 5 to 10 U/L. These values slightly increase at 42°C (to the range of 7–15 U/L). The analysis of conditioned media from cells expressing non-secreted mutants of FGF1 [37,48] demonstrated no release of these mutants at 42°C when LDH activity in the medium did not exceed 20 U/L. In the present study, medium samples were analyzed for LDH activity, and the media were discarded when LDH activity exceeded 15 U/L. Equal amounts of media conditioned by control and heat shocked NIH 3T3 cells transduced with the adenovirus encoding V5-tagged SK1 were collected, treated with 0.1% DTT as described in Materials and Methods, and adsorbed to heparin-Sepharose. The unbound proteins (flow through) were collected and analyzed using immunoprecipitation with antibodies against the V5 epitope. As shown in Figure 1A, the immunoprecipitation demonstrated that SK1 was constitutively released from NIH 3T3 cells at 37°C, and its export was enhanced at 42°C. Figure 1B shows that SK1 released from NIH 3T3 cells did not bind to heparin. Figure 1B also shows the immunoblotting analysis of total cell lysates pertaining to both Figures 1A and 1B. Thus, the results previously obtained using endothelial cells and SK1-transfected 293 cells [20] were confirmed.

In parallel, we transduced SK1 to FGF1 transfectant NIH 3T3 cells. Similarly to cells expressing SK1 exclusively, cells coexpressing FGF1 and SK1 were incubated for 2 hours at 37°C or 42°C, conditioned media were passed through a heparin column, and the flow through was immunoprecipitated with anti-V5 antibodies. Heparin-bound FGF1 and SK1, as well as

SK1 from the flow through, were detected by specific immunoblotting. Co-expression of FGF1, which is a heparin binding protein specifically released at cell stress [25], resulted in a nearly complete disappearance of SK1 from the 37°C flow through (Figure 1A). At this temperature, we also detected only a negligible amount of SK1 adsorbed to heparin (Figure 1B). We suggest that SK1 formed an intracellular complex with FGF1. As a result, while at normal temperature SK1 was efficiently released, FGF1-associated SK1 was retained in the cells. At 42°C, we detected SK1 both in the flow through and in the heparin-bound fraction of the media conditioned by cells coexpressing FGF1 and SK1 (Figure 1A and B). We suggest that the cells co-expressing FGF1 and SK1 release a complex of FGF1 and SK1 at stress, which binds to heparin due to high heparin affinity of FGF1. The presence of SK1 in the flow through at 42°C may be explained by the partial dissociation of the FGF1:SK1 complex in the extracellular compartment. Thus, we concluded that when SK1 was coexpressed with FGF1, it followed the stress-induced FGF1 release pathway.

SK1 is associated with FGF1

The finding that SK1 released during heat shock bound heparin in a FGF1-dependent manner (Figure 1B) is consistent with our previous observations about the other FGF1-binding proteins, S100A13 [49] and p40 Syt1 [47], which are involved in non-classical FGF1 export. Therefore, we evaluated the association between SK1 and FGF1 in the coexpressing cells and in conditioned medium. We performed coimmunoprecipitation experiments utilizing NIH 3T3 cells adenovirally transduced with FGF1:HA and SK1:V5. Cell lysates and conditioned media were obtained from control and heat shocked cells, and after equalization of protein content, the samples were incubated with an anti-HA monoclonal antibody. The immunoprecipitates were resolved by SDS-PAGE, and analyzed by anti-V5 and anti-FGF1 immunoblotting. As shown in Figures 2A and 2B, SK1 and FGF1 were coprecipitated from the cell lysates and from the conditioned medium derived from temperature-stressed cells. As expected, anti-HA antibodies did not precipitate FGF1 or SK1 from the medium conditioned by non-stressed cells. These data indicated that SK1 and FGF1 displayed intracellular association, which resulted in the release of a complex including both FGF1 and SK1.

SK1 knockout abolishes stress-induced FGF1 release

To evaluate the role of SK1 in FGF1 release, we performed experiments with SK1 null mouse embryo fibroblasts [42] immortalized using the large SV40 T antigen, as described in the Materials and Methods. Immortalized fibroblasts from SK1 null and control mice were transduced with the adenovirus encoding FGF1. We observed that SK1 null fibroblasts exhibited a drastic inhibition of FGF1 release under heat shock conditions (Figure 3A). SK1 transduction into the SK1 null cells rescued their ability to exhibit stress-induced FGF1 export (Figure 3C). Thus, SK1 appears to be a critical component of the FGF1 multiprotein release complex. Interestingly, it was reported that SK1 knockout mice do not exhibit an abnormal phenotype, apparently as a result of sufficient S1P levels in these animals [42]. It has been hypothesized that SK2 and possibly other kinases, such as autaxin [50,51] may be alternative sources of S1P production [42]. These data suggest the specificity of the interaction between FGF1 and SK1. Interestingly, we observed that S1P treatment failed to either enhance or inhibit the release of FGF1 from SK1:V5 and FGF1 cotransfectant NIH 3T3 cells both at 37°C and 42°C (data not shown).

SK1 is an avid copper-binding protein, and the coexpression of SK1 and FGF1 rescues heat shock-induced FGF1 export in the presence of TM

The FGF1 release pathway requires intracellular copper, which facilitates the assembly of the FGF1:S100A13:p40Syt1 multiprotein release complex [36]. Because FGF1 [46], S100A13 [36], and p40 Syt1 [36] are copper-binding proteins, and SK1 appears to be a component of

the FGF1 release pathway, we hypothesized that SK1 may also bind copper. We demonstrated that the *in vitro* translation product of the SK1 transcript is an avid copper-binding protein, since it requires 0.5 M imidazole for elution from a copper affinity column (Figure 4A). Notably, p40 Syt1, FGF1, and S100A13 are eluted from this column at 0.05 M imidazole [36]. Furthermore, the analysis of the copper-binding affinity of FGF1 and SK1 from lysates of NIH 3T3 cells coexpressing these proteins demonstrated that a portion of FGF1 was able to gain higher affinity for copper, when co-expressed with SK1 (Figure 4B). These results indicate that the intracellular complex of FGF1 and SK1 exhibited the copper affinity characteristic of SK1.

Because we previously reported that the copper chelator TM is able to repress the heat shock-induced release of FGF1 [36], we investigated whether the constitutive and stress-induced release of SK1 were also copper-dependent. Interestingly, TM treatment was unable to inhibit the release of SK1 at 42°C in the presence or absence of FGF1 (Figure 5A), although a decrease of SK1 release at 37°C in the absence of FGF1 was observed. In addition, the coexpression of SK1 and FGF1 rescued heat shock-induced FGF1 export in the presence of TM (Figure 5B). These data suggest that SK1 may be involved in facilitating the intracellular trafficking of copper needed for the formation of the FGF1 release complex.

SK1 associates with recombinant FGF1 in a cell-free system

Because SK1, like FGF1, S100A13, and p40 Syt1 is a copper-binding protein, and copper is able to mediate *in vitro* interactions between FGF1, S100A13, and p40 Syt1 [36], we questioned whether SK1 is able to form copper-dependent complexes with FGF1. Recombinant FGF1 and SK1 were mixed at equimolar ratio in the presence or absence of 1 mM CuCl₂. After 30 minutes of incubation at 42°C, the reaction products were resolved by non-reducing, limited denaturing (0.1% SDS) SDS-PAGE. We utilized this electrophoretic system, because it enables the resolution and detection by immunoblot analysis of high molecular weight multiprotein aggregates at the top of the stacking gel [36]. FGF1 immunoblotting analysis revealed the appearance of a band at the top of the stacking gel when SK1 was mixed with FGF1 (Figure 6A). Interestingly, however, the appearance of the high molecular weight aggregate was observed even in the absence of 1 mM to associate with CuCl₂, suggesting that SK1 does not require the addition of exogenous copper FGF1 in a cell-free system (Figure 6A). In order to characterize the specificity of the interaction between FGF1 and SK1, we substituted recombinant human FGF1 with the C-terminal portion of osteopontin (OPN) (gift of Dr. L. Liaw, MMCRI, Scarborough, ME), a heparin-binding protein [52] in the reaction mix with SK1. An experiment using increased amounts of SK1 mixed with either 1 µg of FGF1 or C-terminal OPN fragment in copper-free conditions, confirmed the ability of SK1 to form high molecular weight aggregates with FGF1 (Figure 6B, panel a). However, the incubation of SK1 with the C-terminal OPN fragment did not result in the appearance of a high molecular weight aggregate on the top of the stacking gel (Figure 6B, panels b). These results demonstrated that the interaction between FGF1 and SK1 is specific. Because SK1 may facilitate the copper-induced formation of the FGF1 release complex, we sought to assess whether the interaction of SK1 with FGF1 was mediated by endogenous copper bound to SK1. Incubation of recombinant FGF1 at 42°C for 30 minutes in the absence of CuCl₂ together with SK1, which was pretreated with 500 mM imidazole and then extensively dialyzed, strongly reduced the appearance of the FGF1 band resolved at the top of the stacking gel (Figure 6C). Conversely, pretreatment of SK1 with TM in the concentration twice higher than that used in the experiment on rescue of FGF1 release, did not attenuate formation of the complex between recombinant SK1 and FGF1 (Figure 6C). This result indicates that copper ions are very tightly bound to SK1. Collectively, the data on protein complex formation and the observation that the coexpression of SK1 rescued the export of FGF1 when incubated with TM, suggest that SK1

may be involved in the redistribution of intracellular copper required for the assembly of the FGF1 multiprotein release complex.

Discussion

FGF1 contributes to the regulation of cell proliferation, migration, and differentiation during angiogenesis and inflammation. Although FGF1 mediates its biological activity through interactions with cell surface receptors [32], it lacks a conventional signal peptide sequence that is necessary to access the endoplasmic reticulum [25]. However, it was demonstrated that FGF1 is released into the extracellular compartment through a stress- and copper-dependent non-classical pathway. Stress-dependent release of FGF1 requires the formation of a multiprotein complex containing the calcium- and acidic phospholipid-binding proteins, S100A13, and the p40 form of p65 Syt1 [35,47]

SK1 is a regulator of angiogenesis [53,54] through its ability to synthesize S1P [20]. Interestingly, S1P may be involved in the determination of cell fate [55], and may also play a role in the inhibition of apoptosis [56], and in the regulation of the allergic response [57]. Similar to FGF1, SK1 participates in the regulation of cell proliferation, migration, and vascular maturation [7]. As reported earlier, non-classical export of SK1 shares pharmacologic properties with the FGF1 release [20,25,33]. Our data suggest that SK1 can be released through two pathways one of which is stress-independent and unrelated to FGF1, whereas another is stress-dependent and associated with FGF1 secretion. Moreover, SK1 is involved in the regulation of the stress-induced non-classical export of FGF1. Indeed, the expression of FGF1 was able to repress the constitutive release of SK1 from NIH 3T3 cells, and it restricted the export of SK1 to the stress-induced pathway. The observation that SK1 was associated with FGF1 in heat shock-conditioned medium suggests that SK1 participates in the FGF1 multiprotein release complex. Most importantly, the critical role of SK1 in the non-classical stress-induced release of FGF1 was clearly demonstrated in the experiments with SK1 null mouse embryo fibroblasts.

It is noteworthy that the expression of SK1 was able to overcome the TM-induced inhibition of FGF1 release, implying a functional role for SK1 in the copper-dependent assembly of the FGF1 multiprotein release complex. Interestingly, SK1 exhibited an affinity for copper which is much higher than that of FGF1 [46], S100A13 [36], or p40 Syt1 [36]. The observation that SK1 was able to alter the electrophoretic mobility of FGF1 in a cell-free and copper-free system is significant. Indeed, prior electrophoretic analysis of FGF1, S100A13, and p40 Syt1 complex formation revealed a mobility shift reflecting the association of these proteins in response to the presence of exogenous copper [36]. We hypothesize that over expression of SK1 which exhibits a very high copper affinity prevents the inhibitory effect of TM by providing copper needed for the formation of the FGF1 export complex. Based on our results, we anticipate that SK1 may function as an intracellular “copper sink”, and it may be responsible for the distribution of intracellular copper to the polypeptide components of the FGF1 non-classical export pathway. As a result, SK1 contributes to the formation of the multiprotein export complex which contains both FGF1 and SK1.

Interestingly, SK1 is able to interact with acidic phospholipids, particularly phosphatidylserine (pS) and phosphatidylinositol (pI) [45]. It is noteworthy that FGF1 is characterized by the presence of a consensus sequence for acidic phospholipid binding in the carboxy-terminal domain [37,58]. Moreover, the ability of FGF1 [58] to assume a molten globule character in the presence of acidic phospholipids suggests that the FGF1 multiprotein release complex may utilize the flipping of pS or other acidic phospholipids for their export through the plasma membrane [22,59]. Recently, we demonstrated that mutations of lysine clusters located in the carboxy terminal domain of FGF1 interfere both with its release and its ability to destabilize

artificial membranes composed of acidic phospholipids [48]. We suggest that proteins of FGF1 release complex, including SK1, are able to be translocated through cell membrane due to their ability to interact with acidic phospholipids.

In conclusion, the presented results obtained on cell cultures made to express FGF1 and SK1 indicate that the latter is a component of the non-classical FGF1 export pathway. Unfortunately, we were not able to find a cell model with endogenous levels of both FGF1 and SK1 expression sufficient for the secretion studies. However, the experiments with cells derived from SK1 null mice demonstrate the importance of SK1 for FGF1 release. Although the biological significance of SK1 as a component of the FGF1 multiprotein release complex is not fully understood, the biological relevance of ceramide as a mediator of the assembly of this complex was determined. Indeed, the response to injury in the rat aorta is sensitive to TM treatment by its ability to repress the release of IL1 α , FGF1, and S100A13, as well as to reduce the appearance of pS on the surface of cells [60]. Because S1P is a mediator of angiogenic response *in vivo* [10,54,61], it is interesting that SK1, which is responsible for the production of S1P, is a component of the proangiogenic FGF1 release complex. It is not known whether the regulation of tissue response to injury *in vivo* involves the function of S1P generated in the extracellular compartment by SK1 released through the stress-induced pathway in association with FGF1. However, it is likely that at least one of the functions of intracellular cytosolic FGF1 may be to assure that SK1 remains within the cytosol, because its constitutive release may lead to premature angiogenic and inflammatory responses *in vivo*.

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Figure 1.

Release of SK1 in Response to Temperature Stress *In Vitro*. NIH 3T3 cells and FGF1 NIH 3T3 cell transfectants were adenovirally transduced with AdSK1:V5, and subjected to heat shock. After treatment with 0.1% DTT, the conditioned media were adsorbed to and eluted from a heparin-Sepharose column (B), whereas the unbound proteins (flow through) were collected, concentrated, and subjected to immunoprecipitation with anti-V5 antibodies (A) as described in the Materials and Methods. The samples were resolved in a 12% acrylamide SDS-PAGE, and immunoblotted using anti-V5 (A, B top panel) or anti-FGF1 antibodies (B, bottom panel). On the right side of the figure, the densitometric evaluation of SK1 or FGF1 release is presented. The analysis of SK1 and FGF1 expression in the cell lysates (1/10 of total cell lysate loaded) is presented as part of Figure 1B and it pertains to both Figures 1A and 1B. The densitometric values for released proteins were normalized to total SK1 or FGF1 expression levels.

Figure 2.

SK1 Coprecipitates with FGF1. Conditioned media (CM) and cell lysates (Lys) from heat shocked and control NIH 3T3 cell, which were adenovirally transduced with FGF1 or cotransduced with FGF1:HA and SK1:V5, were subjected to immunoprecipitation with a monoclonal anti-HA antibody as described in the Materials and Methods. (A) Anti-HA immunoprecipitates were resolved by 12% acrylamide SDS-PAGE, and subjected to anti-V5 immunoblotting. (B) Anti-HA immunoprecipitates were resolved by 12% acrylamide SDS-PAGE, and subjected to anti-FGF1 immunoblotting.

Figure 4.

(A) SK1 Binds Copper with High Affinity. The SK1:V5 translation product from an *in vitro* translation reaction was adsorbed to a HiTrap chelating column previously loaded with 0.1 M CuCl₂. The column was washed with 1 M NaCl, and eluted with increasing concentrations of imidazole (0.1 mM to 1.0 M) or with 100 mM EDTA. The eluted fractions were subjected to anti-V5 immunoblotting. (B) Copper-binding affinity shift of FGF1 and SK1 upon their coexpression in NIH 3T3 cells. FGF1 NIH 3T3 transfectant cells (panel a), NIH 3T3 cells transduced with AdSK1:V5 (panel b) and FGF1 NIH 3T3 transfectant cells transduced with AdSK1:V5 (panels c and d) were lysed in the EDTA-free NPB buffer (see Materials and Methods). Cell lysates were adsorbed to copper columns and eluted with increased concentrations of imidazole, as described in the Materials and Methods. The eluates were resolved by 12% acrylamide SDS-PAGE, and subjected to anti-FGF1 (panel a and c) or to anti-V5 immunoblotting (panels b and d). On the right side of the figure, the densitometric evaluation of the percentage of SK1 or FGF1 eluted from the copper column at specific imidazole concentrations is presented. The densitometric values of different fractions were normalized to total SK1 or FGF1 adsorbed to the columns.

Figure 5.

Expression of SK1 Rescues the TM-Induced Inhibition of FGF1 Export. AdSK1:V5-transduced NIH 3T3 cells and AdSK1:V5-transduced FGF1 NIH 3T3 cell transfectants were incubated for 18 hours at 37°C in the presence or absence of 250 nM TM. After incubation, the cells were subjected to heat shock for 110 minutes at 42°C in DMEM with or without 250 nM TM. (A) Conditioned media from AdSK1:V5-transduced NIH 3T3 cells, and AdSK1V5-transduced FGF1 NIH 3T3 transfectant cells were subjected to anti-V5 immunoprecipitation and anti-V5 immunoblotting, (B) Conditioned media from either FGF1 NIH 3T3 cell transfectants or AdSK1:V5-transduced FGF1 NIH 3T3 cell transfectants were processed for heparin-Sepharose chromatography, as described in the Materials and Methods. The heparin-Sepharose column was eluted with 1.5 M NaCl, and the fractions were resolved in a 15% acrylamide SDS-PAGE, and subjected to FGF1 immunoblot analysis.

Figure 6.

Interaction Between the Recombinant Forms of SK1 and FGF1 in a Cell-free System. (A). The recombinant FGF1 (100 ng) and SK1 (250 ng) were resuspended in 50 μ l of PBS in the presence or absence of 1 mM CuCl₂, incubated for 30 minutes at 42°C, diluted in non-reducing SDS-PAGE loading buffer, resolved by 12% acrylamide non-reducing/limited denaturing SDS-PAGE [36], and subjected to immunoblotting, as described in the Materials and Methods. Note the multiprotein aggregate band at the top of the stacking gel (arrow) detected by FGF1 (top panel) and SK1 (bottom panel) immunoblot analysis. (B). One μ g of either human recombinant FGF1 or C-terminal portion of mouse recombinant osteopontin (OPN) were resuspended in PBS and mixed with increasing amounts of recombinant SK1 (molar ratio of 0.5, 1, 2.5) in the

absence of exogenous copper, incubated at 42°C for 30 minutes, and processed as described in Figure 6A. The proteins were visualized by immunoblotting with polyclonal antibodies against FGF1 (panel a) and against OPN (panel b and c). (C). Recombinant SK1 was incubated for 30 min at 37°C with or without 500 mM imidazole or 500 μ M TM. After incubation, SK1 was dialyzed against PBS. Complex formation between recombinant SK1 and FGF1 was analyzed as described in Figure 6A.