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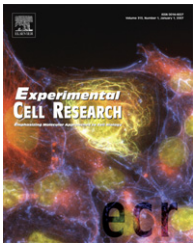

Research Article

Sphingosine kinase 1 is a critical component of the copper-dependent FGF1 export pathway☆

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ABSTRACT

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.

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 G_1–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].

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

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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 proangiogenic and pro-inflammatory polypeptides such as FGF1 [25], FGF2 [26–28], IL1α [29] and IL1β [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], is 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 μg/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 *Hin*dIII and *Pme*I (New England Biolab, Ipswich, MA); and the fragment was subcloned in the multiple cloning site of the adenovirus shuttle vector, pAdlox, using *Hin*dIII and *Sma*I restriction sites. The resulting plasmid was digested with *Sfi*I, 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 ψ5 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 *Eco*RI 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 h 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 h 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 was 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