

# Eukaryotic Elongation Factor 1A Interacts with Sphingosine Kinase and Directly Enhances Its Catalytic Activity\*

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Tamara M. Leclercq<sup>‡§</sup>, Paul A. B. Moretti<sup>‡</sup>, Mathew A. Vadas<sup>‡</sup>, and Stuart M. Pitson<sup>‡§1</sup>

From the <sup>‡</sup>Hanson Institute, Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia 5000, Australia and the <sup>§</sup>School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia

Sphingosine 1-phosphate (S1P) has many important roles in mammalian cells, including contributing to the control of cell survival and proliferation. S1P is generated by sphingosine kinases (SKs), of which two mammalian isoforms have been identified (SK1 and SK2). To gain a better understanding of SK regulation, we have used a yeast two-hybrid screen to identify SK1-interacting proteins and established elongation factor 1A (eEF1A) as one such protein that associates with both SK1 and SK2. We show the direct interaction of eEF1A with the SKs *in vitro*, whereas the physiological relevance of this association was demonstrated by co-immunoprecipitation of the endogenous proteins from cell lysates. Although the canonical role of eEF1A resides in protein synthesis, it has also been implicated in other roles, including regulating the activity of some signaling enzymes. Thus, we examined the potential role of eEF1A in regulation of the SKs and show that eEF1A is able to directly increase the activity of SK1 and SK2 ~3-fold *in vitro*. Substrate kinetics demonstrated that eEF1A increased the catalytic rate of both SKs, while having no observable effect on substrate affinities of these enzymes for either ATP or sphingosine. Overexpression of eEF1A in quiescent Chinese hamster ovary cells increased cellular SK activity, whereas a small interfering RNA-mediated decrease in eEF1A levels in MCF7 cells substantially reduced cellular SK activity and S1P levels, supporting the *in vivo* physiological relevance of this interaction. Thus, this study has established a novel mechanism of regulation of both SK1 and SK2 that is mediated by their interaction with eEF1A.

Sphingosine kinases (SKs)<sup>2</sup> catalyze the phosphorylation of sphingosine to generate the bio-active phospholipid sphingo-

sine 1-phosphate (S1P). S1P affects many biological processes including calcium mobilization, mitogenesis, apoptosis, atherosclerosis, asthma, hypertension, inflammatory responses, and cytoskeleton rearrangement (1). In humans two sphingosine kinases, SK1 and SK2, have been identified (2, 3). These enzymes appear to be the major control elements regulating cellular S1P levels (4) and thus play a crucial role in modulating the effects attributed to S1P.

There is now convincing evidence that SK1 and S1P play roles in tumorigenesis. We have shown that overexpression of SK1 in NIH-3T3 fibroblasts results in neoplastic transformation of these cells (5). Other studies have demonstrated the involvement of SK1 in estrogen-dependent regulation of breast tumor cell growth and survival (6, 7). Furthermore, French *et al.* (8) have shown that SK1 mRNA levels are elevated in a variety of human solid tumors and, importantly, that tumor growth can be reduced *in vivo* by SK inhibitors. Similarly, we have shown that the use of SK inhibitors or a dominant negative SK can block neoplastic cell transformation induced by oncogenic H-Ras (5).

We have established that activation of SK1 can be mediated by phosphorylation at Ser<sup>225</sup> by ERK1/2 (9). This phosphorylation not only results in a 13-fold increase in the catalytic activity of SK1 but is also required for agonist-induced translocation of this enzyme from the cytoplasm to the plasma membrane (9). Our recent studies have shown that this activation of SK1 and its subsequent translocation are one mechanism by which this enzyme is able to induce neoplastic transformation of cells (10). However, the molecular mechanisms regulating the phosphorylation of SK1 or its translocation to the plasma membrane are not currently known. In an attempt to understand the regulatory mechanisms controlling SK1 activity and function, we have performed a yeast two-hybrid screen to identify SK1-interacting proteins. In this study we report that eukaryotic elongation factor 1A (eEF1A) interacts with both SK1 and SK2. Furthermore, we demonstrate that this interaction directly enhances the catalytic activity of these lipid kinases, both *in vitro* and in cells. Thus, this represents a novel level of regulation of both SK1 and SK2 that is mediated by their interaction with eEF1A.

ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; MOPS, 4-morpholinopropanesulfonic acid; HPLC, high pressure liquid chromatography.

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<sup>1</sup> To whom correspondence should be addressed: Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Rd., Adelaide SA 5000, Australia. Tel.: 61-8-8222-3472; Fax: 61-8-8232-4092; E-mail: stuart.pitson@imvs.sa.gov.au.

<sup>2</sup> The abbreviations used are: SK, sphingosine kinase; S1P, sphingosine 1-phosphate; eEF1A, eukaryotic elongation factor 1A; HA, hemagglutinin; PI4K, phosphatidylinositol 4-kinase; rec-SK1, purified recombinant SK1; rec-SK2, purified recombinant SK2; S6K, S6 kinase; PECAM-1, platelet endothelial cell adhesion molecule-1; IB, immunoblotting; GST, glutathione S-transferase; CHO, Chinese hamster ovary; siRNA, small interfering RNA;

## EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—Human embryonic kidney cells (HEK-293T, ATCC CRL-1573) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml), and streptomycin (1.6 mg/ml). The cells were transiently transfected using the calcium phosphate precipitation method (11), harvested 24 h later by scraping into cold PBS, and lysed by sonication (3 watts for 30 s at 4 °C) in extraction buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM EDTA, 10% glycerol, 0.05% Triton X-100, 10 mM β-glycerophosphate, 1 mM dithiothreitol, and protease inhibitors (Complete<sup>TM</sup>; Roche Applied Science). Protein concentrations of cell lysates were determined with Coomassie Brilliant Blue reagent (Sigma) using bovine serum albumin as standard.

Chinese hamster ovary cells (CHO-K1, ATCC CCL-61) were cultured in Ham's F-12 medium (Invitrogen) containing 10% fetal bovine serum, penicillin (1.2 mg/ml), and streptomycin (1.6 mg/ml). Quiescence was achieved by culturing the cells for 7 days at 100% confluency with the medium changed every 3 days. On day 7 quiescent cells were transfected using Lipofectamine<sup>TM</sup> 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were harvested 24 h later and lysed as described above.

Human adenocarcinoma MCF7 cells (ATCC HTB-22) were cultured in Dulbecco's modified Eagle's medium as described above. The cells were transiently transfected with eEF1A2 siRNAs (siRNA-1, 5'-UGGAAGUUCGAGACCACCAAGUACU-3', and siRNA-2, 5'-UGGUCCUUUUGUCAAUACCUCGCA-3') or control siRNA (Invitrogen) using Lipofectamine RNAiMAX<sup>TM</sup> reagent (Invitrogen) according to the manufacturer's specifications, cultured for 72 h, harvested, and lysed as described above.

**Yeast Two-hybrid Screen**—Yeast two-hybrid screening was performed using the Matchmaker Gal4 Two-Hybrid System 3 (Clontech) according to the manufacturer's instructions. Full-length human SK1 cDNA (GenBank<sup>TM</sup> accession number AF200328) (2) was cloned into pGBKT7 in-frame with the Gal4 DNA-binding domain. This bait construct was then transformed into the yeast strain AH107 together with a human leukocyte cDNA library in pACT2. A total of 1 × 10<sup>6</sup> independent clones were screened.

**Cloning of eEF1A1 and eEF1A2**—Primers for PCR amplification of the full-length human eEF1A1 coding region were designed using the published eEF1A1 cDNA sequence (GenBank<sup>TM</sup> accession number NM001402) (12). The eEF1A1 cDNA was amplified from a human foreskin fibroblast cDNA and hemagglutinin (HA) epitope-tagged at the *c*-terminus with primers 5'-CCGGATCCGCCACCATGGGAAAGGAAAAGACTCATA-3' and 5'-GGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGGTATTTAGCCTTCTGAGCTTTCTG-3'. The PCR product was then cloned into pcDNA3 (Invitrogen) for mammalian expression and pGEX4T-1 (GE Health) for bacterial expression following digestion with BamHI and XhoI. Primers for PCR amplification of the eEF1A2 coding region were designed using the published murine

eEF1A2 cDNA sequence (GenBank<sup>TM</sup> accession number NM007906) (13). The murine eEF1A2 cDNA was amplified from mouse brain cDNA and HA epitope-tagged at the *C* terminus with primers 5'-TAGAATTCCGGCCACCATGGGCAAGGAGAAGACACA-3' and 5'-TAGAATTCAAGCGTAATCTGGAACATCGTATGGGTACTTGCCCGCTTTCTGAGC-3'. The PCR product was then cloned into both pcDNA3 and pGEX4T-2 following digestion with EcoRI. Sequencing verified the orientation and integrity of the human eEF1A1 and mouse eEF1A2 cDNA sequences.

**Generation of GST Fusion Proteins**—eEF1A1 and eEF1A2 cDNAs were expressed in *Escherichia coli* JM109 as GST fusion proteins. Overnight cultures were grown with shaking (200 rpm) at 37 °C in Luria broth containing 100 mg/liter ampicillin. The culture was then diluted into fresh Luria broth and grown with shaking at 37 °C to an A<sub>600</sub> level of 0.6–1.0. Expression of the GST fusion proteins was then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside, and the cultures were incubated with shaking at 37 °C for a further 90 min. The bacterial cells were then harvested by centrifugation at 6000 × g for 20 min at 4 °C, resuspended in 20 ml of cold PBS, and lysed by sonication (three pulses of 5 watts for 30 s on ice with 30 s cooling between each pulse). Triton X-100 was then added to a final concentration of 1%, and the lysates were mixed well and then centrifuged at 20,000 × g for 25 min at 4 °C. The resultant clarified bacterial lysate was then incubated with GSH-Sepharose 4B for 2 h at 4 °C with constant mixing. After this time the GSH-Sepharose beads (with bound protein) were pelleted by centrifugation at 3000 × g for 5 min at 4 °C and washed twice in cold PBS. Protein attached to the GSH-Sepharose was quantitated with Coomassie Brilliant Blue staining following SDS-PAGE using bovine serum albumin as standard. These beads were then either used directly in pulldown analysis, or the GST fusion proteins eluted by incubation with cold PBS containing 10 mM GSH for 30 min with constant mixing. GST-SK1 fusion protein were produced as previously described (2).

**Sphingosine Kinase Assays**—Sphingosine kinase activity was routinely determined using D-erythro-sphingosine (Biomol, Plymouth Meeting, PA) and [γ-<sup>32</sup>P]ATP (PerkinElmer Life Sciences) as substrates, as described previously (14). A unit (U) of SK activity is defined as the amount of enzyme required to produce 1 pmol of S1P/min. Substrate kinetics were analyzed using Michaelis-Menten kinetics with the nonlinear regression program Hyper 1.1s.

**In Vitro Phosphorylation of SK1 and eEF1A**—*In vitro* phosphorylation of GST-SK1 was performed while the protein remained bound to the GSH-Sepharose beads by incubating these beads (2 μg of GST-SK1) with 60 units of ERK2 (Calbiochem) and 1 mM ATP in ERK assay buffer (9 mM MOPS, 11 mM β-glycerophosphate, 2.2 mM EGTA, and 0.4 mM sodium orthovanadate) for 60 min at 30 °C. The beads were then washed with 50 mM Tris/HCl buffer containing 150 mM NaCl and 10% glycerol. *In vitro* phosphorylation of His-tagged SK1 (15) in solution was performed as above and then bound to nickel-nitrilotriacetic acid beads (Invitrogen) by mixing at 4 °C for 1 h. The beads were then washed three times in 1 ml of extraction buffer by centrifugation for 5 min at 12,000 × g. To elute the protein, nickel-nitrilotriacetic acid beads were resus-

## Activation of Sphingosine Kinase by eEF1A

pended in extraction buffer lacking dithiothreitol but containing 250 mM imidazole and rotated at 4 °C for 1 h. *In vitro* phosphorylation of GST-eEF1A1 bound to GSH-Sepharose was performed in a similar manner by incubating these beads (containing 1 μg of GST-eEF1A1) with 0.1 units of S6 kinase (Upstate Biotechnology, Inc.), 3.5 mM [ $\gamma$ -<sup>32</sup>P]ATP (70 nCi/μl) in buffer (7 mM MOPS, 0.1 mM EDTA, 4 μM β-glycerophosphate, 0.2 μM dithiothreitol, 0.15 mM orthovanadate, 0.9 mM EGTA, pH 7.4) for 30 min at 37 °C. The beads were then washed three times with cold PBS. *In vitro* phosphorylation of GST-eEF1A1 in solution was performed using 1 μg of purified GST-eEF1A1 and 50 ng of S6 kinase in 20 mM MOPS, pH 7.2, containing 5 μM EDTA, 0.01% glycerol, 2 μg/ml bovine serum albumin, 1 mM ATP, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM orthovanadate, 5 mM EGTA. The reactions were incubated at 37 °C for 30 min with agitation. Following incubation time the recombinant proteins were immediately used for *in vitro* SK1 activity assays.

**Immunoprecipitation and Immunoblotting**—Lysates from cells expressing the FLAG-tagged SK1 alone and/or in combination with HA-tagged eEF1A isoforms were centrifuged at 13,000 × *g* for 10 min at 4 °C to remove insoluble material. Anti-HA monoclonal antibodies (Sigma), M2 anti-FLAG monoclonal antibodies (Sigma), or rabbit anti-SK1 antibodies (9) were added to the lysates and incubated at 4 °C for 3 h with constant agitation. The immune complexes were then captured by incubation with protein A-Sepharose (Amersham Biosciences) for 3 h at 4 °C, washed with cold extraction buffer, and subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. SK1 was detected with either the monoclonal M2 anti-FLAG antibody (Sigma) or polyclonal chicken or rabbit anti-hSK1 antibodies (9). eEF1A1 was detected with either anti-HA antibodies (12CA5; Sigma) or anti-eEF1A antibodies (Upstate Biotechnology, Inc.). The immunocomplexes were detected with horseradish peroxidase-conjugated anti-mouse (Pierce), anti-rabbit (Pierce), or anti-chicken IgG (IMVS, Adelaide, Australia) using an enhanced chemiluminescence kit (ECL, Amersham Biosciences). α-Tubulin was employed as a loading control using anti-α-tubulin antibodies (Abcam, Cambridge, UK).

**GST Fusion Protein Binding Analyses**—Lysates from transiently transfected HEK-293T cells were incubated with GSH-Sepharose beads containing 1 μg of either GST, GST-eEF1A, or GST-SK1 for 2 h at 4 °C with constant mixing. The beads were then washed three times in 25 mM Tris/HCl, pH 7.4, containing 75 mM NaCl, and 10% glycerol and subjected to SDS-PAGE, and associated proteins were detected by Western blotting with either anti-FLAG or anti-HA antibodies. Interaction of GST-eEF1A1 with purified recombinant SK1 (rec-SK1) or SK2 (rec-SK2) was performed in a similar manner with GST-eEF1A1 bound to GSH-Sepharose incubated with 1 μg of His-tagged rec-SK1 (15) or rec-SK2 (14) for 3 h at 4 °C with constant mixing. The beads were then washed as described above and subjected to SDS-PAGE, and associated proteins were detected by Western blotting with anti-His antibodies (Santa Cruz Biotechnology).

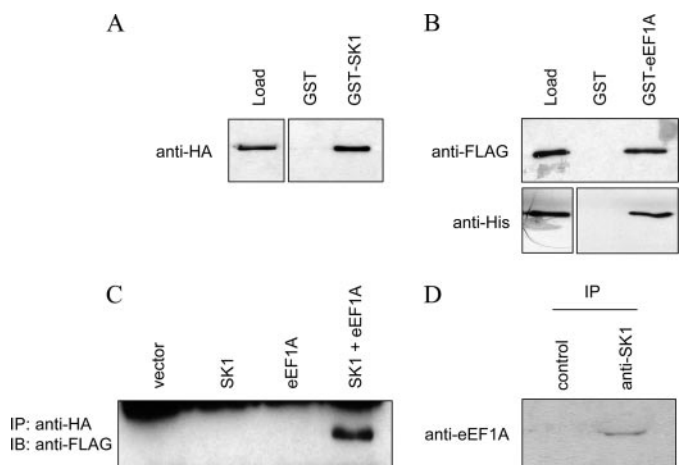
**Lipid Determinations**—S1P levels were measured using fluorescent derivatization followed by HPLC, essentially as

described previously (16). Briefly, S1P was extracted from 100 μl of serum using a two-step Bligh-Dyer extraction, initially under alkaline conditions where S1P largely partitions to the aqueous phase and then under acidic conditions, where S1P partially partitions to the organic phase. C17-S1P (Avanti Polar Lipids) was used as an internal standard. The organic phase was then evaporated, and the dried S1P pellets were dissolved in 275 μl of methanol/70 mM K<sub>2</sub>HPO<sub>4</sub> (3:1). Fluorescent derivatization of S1P was achieved by the addition of 25 μl of a derivatization mixture containing 5 mg *o*-phthalaldehyde, 100 μl of ethanol, 5 μl of β-mercaptoethanol in 5 ml of 3% boric acid, pH 10.5. The mixture was then incubated at room temperature for 15 min prior to HPLC analysis. The samples (100 μl) were applied to an X-bridge C18 column (Waters, Milford, MA) equilibrated with methanol/70 mM K<sub>2</sub>HPO<sub>4</sub> (3:1) using a flow rate of 1.5 ml/min. S1P was then eluted from the column with a 10-min linear gradient to methanol, with fluorescence measured at emission and excitation wavelengths of 455 and 340 nm, respectively. The resulting profiles were evaluated using the Millennium32 Chromatography Manager software (Waters). Cellular sphingosine levels were determined as described previously (17).

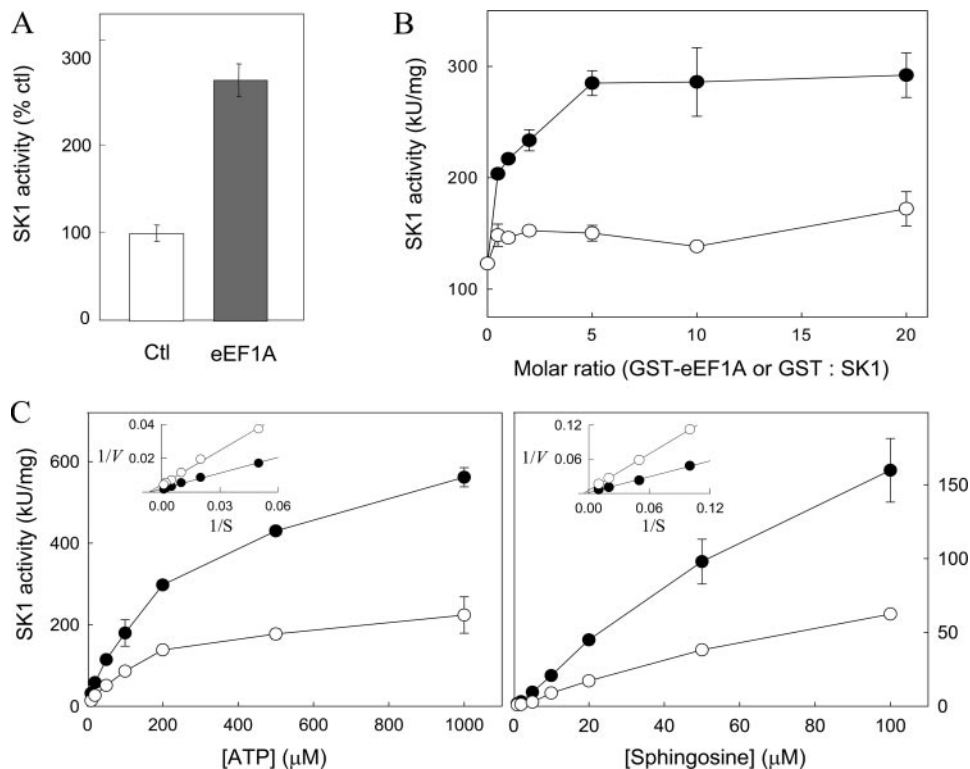
## RESULTS

**eEF1A1 Is a SK1-interacting Protein**—In an attempt to understand the mechanisms regulating the activity and function of SK1, we performed a yeast two-hybrid screen to identify proteins that interact with SK1. One partial cDNA that was isolated in this screen encoded the C-terminal 312 amino acids of eEF1A1.

To confirm the interaction between eEF1A1 and SK1, bacterial and mammalian expression constructs encoding the full-length eEF1A1 cDNA were generated by PCR from human foreskin fibroblast cDNA. The interaction of eEF1A1 with SK1 was first assessed by pulldown experiments using GST-SK1 or GST alone bound to GSH-Sepharose and lysates from HEK-293T cells expressing HA epitope-tagged eEF1A1 (Fig. 1A). These results demonstrated a specific interaction of eEF1A1 with GST-SK1 and not with GST alone. Reverse pulldown experiments were also performed using GST-eEF1A1 or GST alone bound to GSH-Sepharose and lysates from HEK-293T cells expressing FLAG epitope-tagged SK1 (Fig. 1B). Again, the results demonstrated the specific interaction of GST-eEF1A1 with SK1. In similar experiments, purified recombinant SK1 (rec-SK1) also associated with GST-eEF1A1, demonstrating that the interaction of SK1 and eEF1A1 is direct and not mediated by another associated protein. To further confirm the interaction between eEF1A1 and SK1, co-immunoprecipitation studies were performed on lysates from HEK-293T cells co-expressing HA-tagged eEF1A1 and FLAG-tagged SK1. Consistent with the results of the pulldown experiments, the presence of SK1 in the anti-HA (eEF1A1) immunocomplex was observed (Fig. 1C). Finally, we were able to demonstrate the physiological interaction between SK1 and eEF1A1 through the co-immunoprecipitation of the endogenous proteins in untransfected



**FIGURE 1. eEF1A1 interacts with SK1.** *A*, GST-SK1 bound to GSH-Sepharose was incubated with lysates from HEK-293T cells expressing HA-tagged eEF1A1. eEF1A1 pulled down by the GST-SK1 was detected by IB with anti-HA. *B*, GST-eEF1A1 bound to GSH-Sepharose was incubated with lysates from HEK-293T cells expressing FLAG-tagged SK1. SK1 associated with GST-eEF1A1 was detected by immunoblotting (IB) with anti-FLAG antibodies (upper panel). GST-eEF1A1 bound to GSH-Sepharose was incubated with rec-SK1 and SK1 associated with GST-eEF1A1 was detected by IB with anti-His antibodies (lower panel). *C*, lysates from HEK-293T cells co-transfected with FLAG-tagged SK1 and HA-tagged eEF1A1 were immunoprecipitated (IP) with anti-HA and SK1 present in the immunocomplexes detected by IB with anti-FLAG antibodies. *D*, lysates from untransfected HEK-293T cells were incubated with chicken anti-SK1 to IP the endogenous SK1, and then endogenous eEF1A present in the immunocomplexes was detected by IB with anti-eEF1A antibodies.



**FIGURE 2. eEF1A1 enhances SK1 activity *in vitro*.** *A*, the effect of purified GST-eEF1A1 (eEF1A) or GST control (Ctl) on the catalytic activity of purified rec-SK1 was determined by *in vitro* SK assay. GST-eEF1A1 and GST were added to rec-SK1 in 10-fold molar excess. The data are the means  $\pm$  S.E. of one representative experiment of four, each performed in duplicate. *B*, the effect of GST-eEF1A1 (closed circles) or GST control (open circles) on the catalytic activity of rec-SK1 was assessed over a range of increasing concentrations of GST-eEF1A1 or GST. The data are the means  $\pm$  S.E. from three independent experiments each performed in duplicate. *C*, substrate kinetics with ATP and sphingosine for SK1 in the presence of 10-fold molar excess of GST-eEF1A1 (closed circles) or GST control (open circles).  $K_m$  values for ATP were  $89 \pm 9$  and  $106 \pm 13 \mu\text{M}$  for GST and GST-eEF1A1, respectively, whereas  $K_m$  values for sphingosine were  $24 \pm 5$  and  $26 \pm 3 \mu\text{M}$ , respectively.  $k_{\text{cat}}$  values for SK1 in the presence of GST and GST-eEF1A1 were  $124 \pm 14$  and  $304 \pm 26 \text{ s}^{-1}$ , respectively. All of the data shown are the means  $\pm$  S.E. of two independent experiments each performed in duplicate.

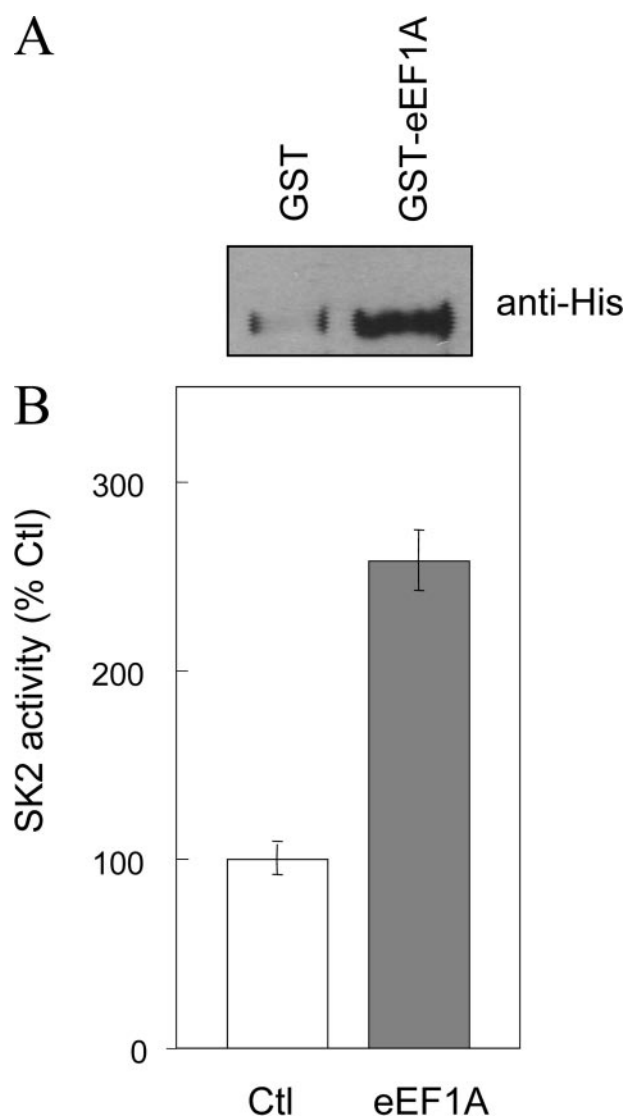
HEK-293T cell lysates (Fig. 1D) using anti-SK1 antibodies to co-immunoprecipitate eEF1A1.

**eEF1A1 Directly Enhances SK1 Activity *In Vitro***—Although the canonical role for eEF1A1 is in polypeptide elongation during protein synthesis (18) various other apparently unrelated cellular functions have been attributed to eEF1A1, including roles in signal transduction, cytoskeletal organization, apoptosis, and oncogenic transformation (19–21). Because eEF1A1 has been shown previously to directly activate phospholipase C $\gamma$  and phosphatidylinositol 4-kinase (PI4K) (22), we hypothesized that it may also have an effect on the activation of SK1. Thus, we examined the effect of purified recombinant GST-eEF1A1 or GST alone on the catalytic activity of rec-SK1 *in vitro*. Although GST alone had no effect on SK1 activity under these conditions, GST-eEF1A1 enhanced the catalytic activity of SK1 2.7-fold (Fig. 2A). Furthermore, this effect was observed to be dose-dependent, with SK1 activity increasing as the concentration of eEF1A1 increased, reaching a maximum with a 5-fold molar excess of eEF1A1 to SK1 (Fig. 2B). This effect was not as a result of eEF1A1 having any intrinsic ability to phosphorylate sphingosine, nor was it due to eEF1A1 increasing the stability of rec-SK1 in the enzyme assay, because the enzyme is stable under these assay conditions as demonstrated by linear reaction kinetics (data not shown). We next examined

the effect of eEF1A1 on SK1 substrate kinetics. The results show that the  $K_m$  values of SK1 for both sphingosine and ATP were unaltered by the presence of eEF1A1 (Fig. 2C). In contrast, the  $k_{\text{cat}}$  for SK1 was increased 2.5-fold by the presence of eEF1A1 (Fig. 2C). Taken together these results indicate that eEF1A1 does not increase the affinity of SK1 for its substrates but rather has a direct stimulatory effect on the catalytic rate of SK1.

**eEF1A1 Interacts with and Enhances SK2 Activity**—Because eEF1A1 interacts with SK1 and has a direct effect on its activity, we next examined its effect on the other human SK isoform, SK2. The interaction between eEF1A1 and SK2 was investigated using GST-eEF1A1 or GST alone bound to GSH-Sepharose and purified rec-SK2. Like SK1, SK2 was able to specifically interact with GST-eEF1A1 (Fig. 3A).

Having demonstrated that eEF1A1 and SK2 interact, we next examined the effect of the interaction on the catalytic activity of SK2. Purified GST-eEF1A1 and purified rec-SK2 were incubated *in*



**FIGURE 3. eEF1A1 interacts with and activates SK2 *in vitro*.** *A*, purified recombinant His-tagged SK2 (rec-SK2) was incubated with GST-eEF1A1 or GST alone control bound to GSH-Sepharose. SK2 associated with GST-eEF1A1 or GST control was detected by IB with anti-HIS antibodies. *B*, The effect of purified GST-eEF1A1 (eEF1A) or GST control (Ctl) on the catalytic activity of purified rec-SK2 was determined by *in vitro* SK assay. GST-eEF1A1 and GST were added to SK2 in 10-fold molar excess. The data are the means  $\pm$  S.E. of three experiments each performed in duplicate.

*in vitro*, and the resultant SK activity was measured. As with SK1, GST-eEF1A1 was shown to increase SK2 activity by ~2.6-fold (Fig. 3*B*).

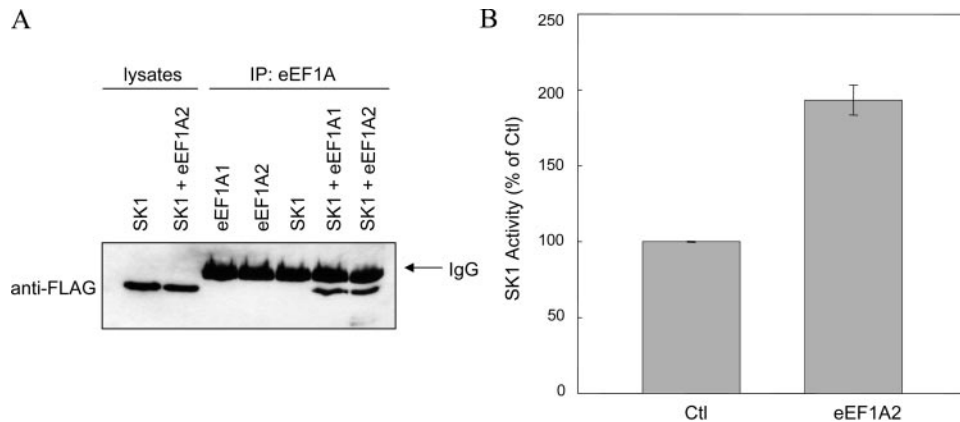
**eEF1A2 Is a SK1-interacting Protein**—Two eEF1A isoforms exist in humans, eEF1A1 and eEF1A2 (18). Although the sequence similarity between these two proteins is extremely high (92% polypeptide sequence identity) (20), some differences have been observed in their distribution within human tissues (18, 20), with eEF1A1 ubiquitously expressed, and eEF1A2 only present in heart, brain, and skeletal muscle cells (20). Our yeast two-hybrid screen and the analyses outlined above identified eEF1A1 as a SK1-interacting protein. Given the sequence homology of the two proteins, we next examined whether eEF1A2 also interacted with SK1. Co-immunoprecipitations were performed using lysates from HEK-293T cells co-

expressing HA-tagged eEF1A2 and FLAG-tagged SK1, demonstrating that, as with eEF1A1, eEF1A2 also associates with SK1 (Fig. 4*A*). In agreement with our eEF1A1 results, *in vitro* studies also showed that GST-eEF1A2 was also able to increase the activity of rec-SK1 2.4-fold (Fig. 4*B*).

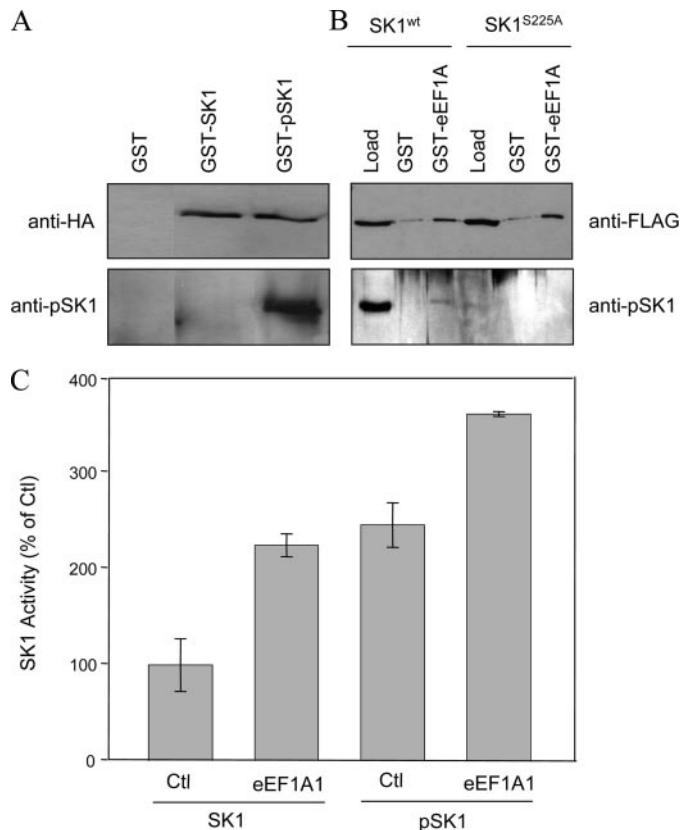
**Interaction of SK1 and eEF1A1 Is Not Regulated by Phosphorylation**—The interaction between eEF1A1 and various proteins is known to be regulated by phosphorylation, either of eEF1A1 (23) or of its target proteins (19, 22). Therefore, we examined whether the phosphorylation state of SK1 or eEF1A1 affected their interaction. We first investigated the effect of SK1 phosphorylation by pull-down experiments using GST-eEF1A1 bound to GSH-Sepharose and lysates from HEK-293T cells overexpressing either wild type SK1, known to be phosphorylated (9), or its nonphosphorylatable counterpart SK1<sup>S225A</sup> (9). The results of the pull-down experiments showed that both forms of SK1 interacted with eEF1A1 to a comparable extent (Fig. 5*B*). To confirm this result, we also phosphorylated GST-SK1 bound to GSH-Sepharose using recombinant ERK2. ERK2 is known to specifically phosphorylate SK1 at Ser<sup>225</sup> *in vitro*, which appears to be the only physiological phosphorylation site in this protein (9). This phosphorylated GST-SK1 was then used for pull-down analyses with lysates from HEK-293T cells expressing HA-tagged eEF1A1. Both nonphosphorylated GST-SK1 and phosphorylated GST-SK1 interacted with eEF1A1 in a comparable manner (Fig. 5*A*). Together, these results show that, unlike some other eEF1A1-associated proteins, the phosphorylation state of SK1 does not affect its ability to interact with eEF1A1. Furthermore, examination of the effect of GST-eEF1A1 on the catalytic activity on *in vitro* phosphorylated SK1 showed that eEF1A1 enhanced the activity of phosphorylated and nonphosphorylated SK1 equally (Fig. 5*C*). Thus, consistent with our previous studies, *in vitro* phosphorylation of SK1 increased its catalytic activity (9), but eEF1A1 could further enhance this activity.

Next, we examined the effect of eEF1A1 phosphorylation on its ability to interact with SK1. eEF1A1 is known to be phosphorylated *in vitro* by S6 kinase (S6K) (20), protein kinase C $\delta$  (24), and Rho-associated kinase (19). Because S6K phosphorylates eEF1A1 at multiple sites including those sites phosphorylated by protein kinase C $\delta$  (20) and Rho-associated kinase (19), GST-eEF1A1 bound to GSH-Sepharose was phosphorylated with S6K (Fig. 6*A*) and then used in pull-down experiments with rec-SK1 and rec-SK2. The results indicate that both nonphosphorylated and phosphorylated GST-eEF1A1 is able to interact with SK1 and SK2 to a similar extent (Fig. 6*B*). Similarly, GST-eEF1A1 phosphorylated in solution was able to activate rec-SK1 to the same extent as nonphosphorylated GST-eEF1A1 (Fig. 6*C*). Therefore, the results show that unlike the situation with some other eEF1A1 interacting proteins, the phosphorylation state of eEF1A1 or SK1 has no effect on the interaction of these two proteins and the subsequent increase in SK activity.

**eEF1A1 Overexpression in Quiescent Cells Enhances Endogenous SK Activity**—Despite the *in vitro* effects of eEF1A on the SKs, no increase in cellular SK activity was observed when eEF1A1 was overexpressed in either proliferating HEK-293T or CHO cells (data not shown). These results were perhaps not surprising because eEF1A has been reported to be one of the



**FIGURE 4. eEF1A2 interacts with SK1 and increases its activity.** A, HEK-293T cells were co-transfected with FLAG-tagged SK1 and either HA-tagged eEF1A1 or eEF1A2. eEF1A1 or eEF1A2 were immunoprecipitated (IP) from the lysates using anti-HA antibodies and the SK1 present in the immunocomplexes detected using anti-FLAG antibodies. B, the effect of purified GST-eEF1A2 (eEF1A2) or GST control (Ctl) on the catalytic activity of rec-SK1 was determined by *in vitro* SK assay. GST-eEF1A2 and GST were added to SK1 in 10-fold molar excess. The data are the means  $\pm$  S.E. of two experiments each performed in duplicate.



**FIGURE 5. Phosphorylation of SK1 does not alter binding to eEF1A1.** A, GST-SK1 bound to GSH-Sepharose was phosphorylated using ERK2, as assessed through IB with anti-phospho-SK1 antibodies. GST-SK1 and GST-phospho-SK1 (GST-pSK1) were then incubated with lysates from HEK-293T cells expressing HA-eEF1A1. eEF1A1 associated with GST-SK1 and GST-pSK1 was detected by IB with anti-HA antibodies. B, GST-eEF1A1 bound to GSH-Sepharose was incubated with lysates from HEK-293T cells expressing FLAG-tagged wild type SK1 ( $SK1^{wt}$ ) or the nonphosphorylatable version of SK1 ( $SK1^{S225A}$ ) and the SK1 associated with GST-eEF1A1 detected by IB with anti-FLAG antibodies. Phospho-SK1 present in the lysates and pull-downs were determined by IB with anti-pSK1 antibodies. C, purified GST-SK1 was phosphorylated *in vitro* by ERK2 and the effect of purified GST-eEF1A1 (eEF1A1) or GST control (Ctl) on its catalytic activity determined by *in vitro* SK assay. GST-eEF1A1 and GST were added to GST-SK1 in 10-fold molar excess. The data represent the means  $\pm$  S.E. for two experiments each performed in duplicate.

most highly expressed proteins in actively dividing cultured cells (25). Thus, the high endogenous levels of eEF1A in these proliferating cells is likely to already have an effect on cellular SK activity in the absence of further eEF1A1 overexpression. In quiescent cells, however, which represent the majority of cells in an adult mammal, eEF1A expression is substantially reduced (26). Thus, we examined the effect of eEF1A overexpression on cellular SK activity in quiescent cells. Consistent with previous reports (26), the growth of CHO cells to quiescence resulted in a substantial decrease in the levels of endogenous eEF1A protein compared with that observed in actively

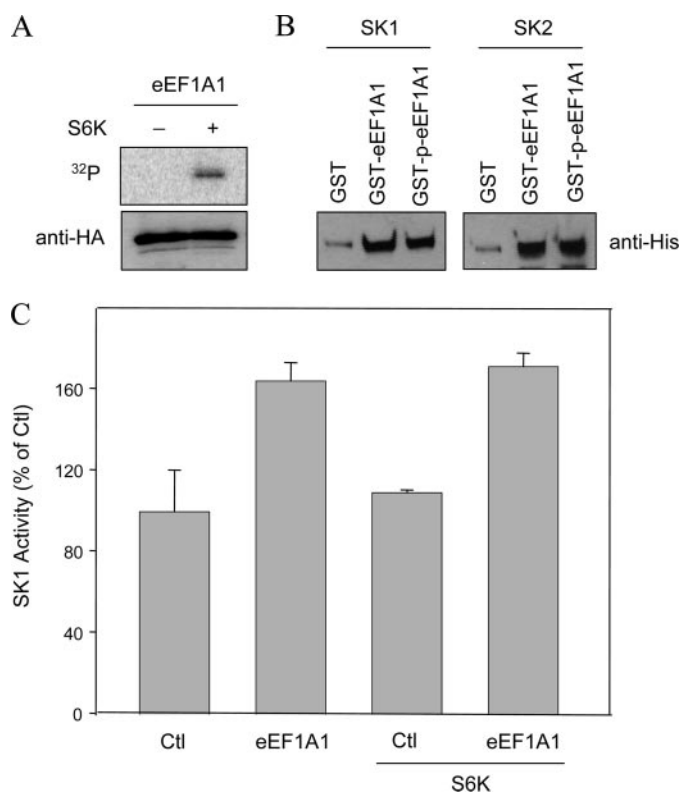
proliferating cells (Fig. 7A). Subsequent overexpression of eEF1A1 in these quiescent cells, to even modest levels (Fig. 7B), resulted in an increase in cellular SK activity (Fig. 7C), indicating a potential physiological role for eEF1A in the regulation of SK activity *in vivo*.

*siRNA-mediated Down-regulation of eEF1A2 Decreases Endogenous SK Activity and S1P Levels*—To further examine a physiological role for eEF1A in the regulation of cellular SK activity, we employed a siRNA knockdown approach. Design of siRNAs for knockdown of eEF1A1 expression is problematic because of the presence of a large number of potential eEF1A1 pseudogenes (27). No such difficulties exist for eEF1A2, however, and recent reports have demonstrated that this is the major eEF1A isoform in MCF7 cells (28). Thus, MCF7 cells were transfected with either eEF1A2 siRNA or control siRNA and then examined for the level of total eEF1A (1 and 2) protein in the cell lysates by immunoblot analysis with a pan-eEF1A antibody 72 h later. The results showed effective siRNA knockdown of total eEF1A levels in these cells to less than 40% of that observed in control cells (Fig. 8A). SK assays performed on these lysates demonstrated a remarkable decrease in SK activity (Fig. 8B), further suggesting a physiological role for eEF1A in the regulation of endogenous SK activity. Similar results were also demonstrated with a second eEF1A2 siRNA (data not shown). Subsequent analysis of cellular S1P in response to eEF1A knockdown also showed a substantial decrease in the levels of this phospholipid (Fig. 8C), further supporting the apparent physiological role of eEF1A in regulating cellular SK activity. Notably, this decrease in endogenous SK activity and S1P levels was not due to a nonspecific siRNA-induced decrease in cellular SK protein (Fig. 8A) or sphingosine levels (data not shown).

**DISCUSSION**

Activation of SK and the subsequent increases in cellular S1P levels are involved in modulating many cell signaling pathways and have been implicated in the development of the neoplastic cell phenotype (10, 29–31). We have previously reported that one mechanism regulating the cellular activity of SK1 involves

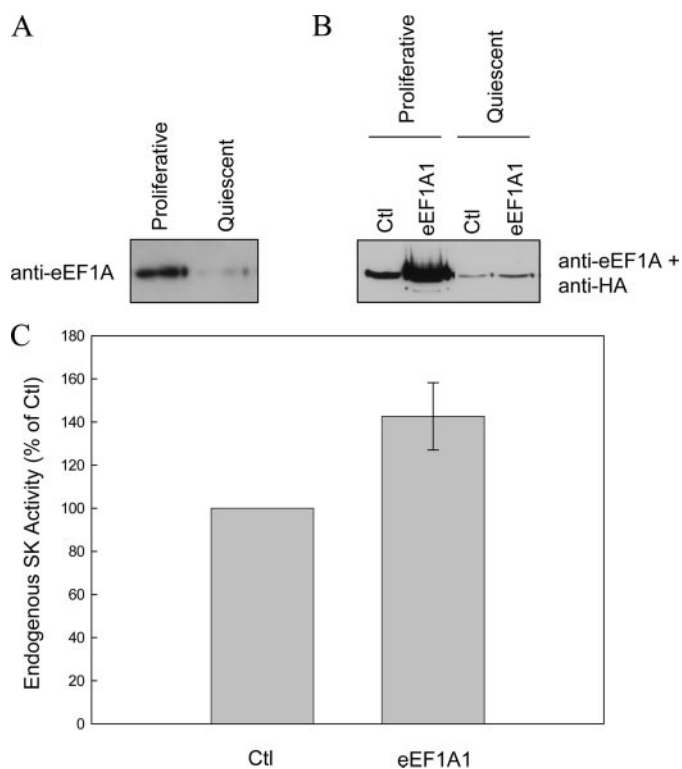
## Activation of Sphingosine Kinase by eEF1A



**FIGURE 6. Phosphorylation of eEF1A1 does not alter binding to SK1 or SK2.** A, GST-eEF1A1 bound to GSH-Sepharose was incubated with S6K in the presence of [ $\gamma$ - $^{32}$ P]ATP, and the phosphorylation of GST-eEF1A1 was detected using phosphorimaging (upper panel). The lower panel (anti-HA) shows equal input of HA-tagged GST-eEF1A1. B, rec-SK1 and rec-SK2 were incubated with GST-eEF1A1 and GST-phospho-eEF1A1 bound to GSH-Sepharose and the SK1/2 associated with the GST fusion proteins detected by IB with anti-HIS antibodies. C, GST-eEF1A1 in solution was phosphorylated using S6K. The effect of this GST-phospho-eEF1A1 on the catalytic activity of rec-SK1 was then determined by *in vitro* SK assay and compared with the effect of nonphosphorylated GST-eEF1A1 or GST control (Ctl). GST-eEF1A1 and GST were added to SK1 in 10-fold molar excess. The data are the means  $\pm$  S.E. of two independent experiments performed in duplicate.

its agonist-induced phosphorylation at Ser<sup>225</sup> by ERK1/2 (9). In the current study we report an alternate novel mechanism of regulating SK activity through the interaction with eEF1A, which results in a direct enhancement of the catalytic activity of both SK1 and SK2.

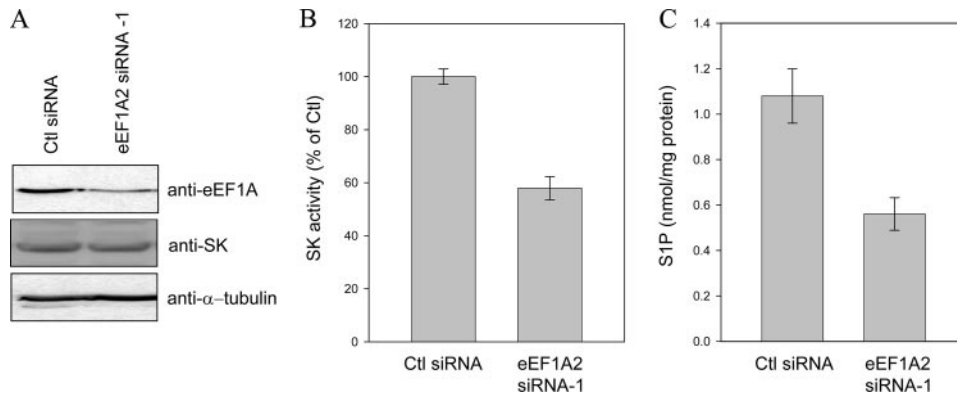
In humans two isoforms of eEF1A have been defined, eEF1A1 and eEF1A2 (19, 20). The canonical role for eEF1A is as part of the nonribosomal translation elongation machinery that facilitates peptide chain elongation during mRNA translation (32). However, eEF1A also has a wide array of other “moonlighting” functions in the cell, including regulating the activity of signaling enzymes (19), involvement in apoptosis and cytoskeletal reorganization through regulating bundling and severing of F-actin and microtubules (19), and an apparent role in oncogenesis (19–21). In keeping with these alternate functions of eEF1A, we now demonstrate that both eEF1A1 and eEF1A2 can directly enhance the catalytic activity of SK1 and SK2 via their interaction with these proteins. Although the molecular mechanism whereby eEF1A mediates this enhanced SK activity is not currently known, substrate kinetic analysis indicates that this effect results from a direct increase in the catalytic efficiency of SK, rather than an alteration in its affinity



**FIGURE 7. Overexpression of eEF1A1 in quiescent cells increases endogenous SK activity.** A, endogenous eEF1A protein levels in proliferative and quiescent CHO cell lysates were examined by IB with anti-eEF1A antibodies. The lanes were loaded with equal total protein. B, proliferative and quiescent CHO cells were transfected with vector encoding HA-tagged eEF1A1 or empty vector (Ctl), and the total level of eEF1A protein in the cell lysates were determined by IB with anti-eEF1A and anti-HA antibodies. C, lysates from quiescent CHO cells expressing HA-tagged eEF1A1 were analyzed for endogenous SK activity and compared with empty vector transfected cells (Ctl). The data are the means  $\pm$  S.D. or five independent experiments ( $p < 0.05$ ).

for either ATP or sphingosine. Interestingly, SK1 phosphorylation also results in increased catalytic efficiency, without any notable changes in the  $K_m$  values for either substrate (9). This initially suggested the possibility that similar molecular mechanisms enhance SK activity mediated by both the interaction with eEF1A and phosphorylation. Our subsequent studies, however, indicate that this may not be the case because the catalytic activity of phosphorylated SK1 can be further enhanced by the presence of eEF1A1. Thus, this suggests that at least two independent mechanisms for the regulation of SK1 exist, one that employs phosphorylation, and one involving interaction with eEF1A.

eEF1A1 and its plant homologue PIK-A49 have been reported to directly enhance the catalytic activity of phospholipase C $\gamma$ 1 and PI4K (23, 33, 34), respectively. Interestingly, however, PIK-A49 appears to modulate PI4K via a different mechanism to that displayed by eEF1A on SK because Yang and Boss (23) reported this protein enhanced the affinity of PI4K for ATP. Somewhat surprisingly, in light of their earlier studies reporting that PIK-A49 induced enhanced PI4K activity in the presence of excess substrates (33), this group were unable to see an effect of PIK-A49 on the  $V_{max}$  of PI4K (23). Nevertheless, recent studies demonstrated the ability of eEF1A to enhance mammalian PI4K activity in MCF7 cells (28).



**FIGURE 8. siRNA-mediated down-regulation of eEF1A2 in MCF7 cells decreases endogenous SK activity and S1P levels.** *A*, siRNAs directed against eEF1A2 were transfected into MCF7 cells, and the decrease in the overall level of eEF1A protein was determined in the cell lysates by IB with anti-eEF1A antibodies (*top panel*). Endogenous SK protein was determined by IB with rabbit anti-SK antibodies (*center panel*), and even loading was confirmed by IB with anti- $\alpha$ -tubulin (*bottom panel*). The data shown are for eEF1A2 siRNA-1. Similar results were observed for siRNA-2 (data not shown). *B*, lysates from MCF7 cells transfected with eEF1A2 siRNA (*eEF1A2 siRNA-1*) or control siRNA (*Ctl siRNA*) were analyzed for endogenous SK activity as previously described. The data are the means  $\pm$  S.E. for five independent experiments each performed in duplicate. *C*, lysates from MCF7 cells transfected with eEF1A2 siRNA-1 or control siRNA were analyzed for endogenous levels of S1P. The data are the means  $\pm$  S.E. of two independent experiments performed in duplicate.

Because eEF1A is a highly abundant protein in the cell, we examined the possible mechanisms regulating its effects on SK activity. The interaction of eEF1A with some proteins, including phospholipase C $\gamma$ 1 (34) and zinc finger protein 1 (35), are enhanced by cell exposure to epidermal growth factor, although the mechanism whereby this occurs is currently unknown. The finding that eEF1A is multiply phosphorylated in response to epidermal growth factor and other growth factors has led to suggestions that this may regulate its agonist-induced protein interactions (19). Certainly, phosphorylation of eEF1A appears important in its binding to actin (36), as well as its interaction and activation of PI4K (23). In contrast, epidermal growth factor-induced phosphorylation of phospholipase C $\gamma$ 1 has been proposed to regulate its interaction with eEF1A (34). Thus, phosphorylation appears a common process controlling the effects of eEF1A on its cellular targets. Examination of the involvement of phosphorylation of either eEF1A1 or SK1 in their interaction, however, failed to demonstrate this as a likely regulatory mechanism for the effect of eEF1A on cellular SK activity.

Although there is no evidence for post-translational modifications of eEF1A regulating its effects on SK activity, one likely alternative is that the level of eEF1A protein itself regulates cellular SK activity. It is notable that rapidly growing cultured cells exhibit a large increase in eEF1A levels compared with that seen in tissues (26, 37). Indeed, in an attempt to deduce a physiological role for eEF1A, we ectopically expressed eEF1A1 in HEK-293T and CHO cells to determine its effect on cellular SK activity. No effect was observed, presumably because of the already extremely high abundance of eEF1A in these proliferating cells (25, 26). However, in quiescent cells, where endogenous eEF1A levels are substantially decreased, overexpression of eEF1A resulted in a significant increase in cellular SK levels. Furthermore, decreasing the levels of endogenous eEF1A via siRNA in MCF7 cells resulted in a dramatic reduction in both endogenous SK activity and cellular S1P levels. Together, these

data strongly suggest eEF1A may be a biologically relevant regulator of SK *in vivo*. Thus, enhanced eEF1A expression that coincides with cell proliferation may not only allow for increased protein translation but also enhance cellular SK activity to provide further proliferation and survival signals.

A number of other SK1-interacting proteins have been recently identified, including platelet endothelial cell adhesion molecule-1 (PECAM-1) (38),  $\delta$ -catenin (39), tumor necrosis factor  $\alpha$  receptor associated factor 2 (40), a protein kinase A anchoring protein-like protein SKIP1 (41), aminoacylase 1 (42), RPK118 (43), Lyn kinase (44), Fyn kinase (45), and LIM-only factor FHL2/SLIM3 (46). Like eEF1A,

several of these proteins have been reported to enhance SK1 activity. For example,  $\delta$ -catenin has been shown to interact with SK1 and directly increase its activity *in vitro* (39). Similarly, the Src family kinases, Lyn and Fyn, have also been reported to interact with and activate SK1 (44, 45), while also causing recruitment of SK1 to the Fc $\epsilon$ RI antigen receptor to modulate mast cell functions such as chemotaxis and degranulation (44, 45). Because Lyn and Fyn do not phosphorylate SK1 this lead to speculation that these tyrosine kinases most likely enhance SK1 activity through inducing a conformational change in the protein directly by the protein-protein interaction (44). The activity of SK1 (and SK2) may also be enhanced by eEF1A via a similar mechanism. Because the polypeptide sequences of the two eEF1A isoforms do not show any discernible similarity to Lyn, Fyn, or  $\delta$ -catenin, however, it is difficult to predict whether all of these proteins act on SK1 by the same mechanism.

Interestingly, whereas several proteins have now been shown to enhance SK1 activity, overexpression of some other SK1-interacting proteins, such as PECAM-1 (38), SKIP1 (41), and aminoacylase 1 (42) result in a modest decrease in cellular SK activity. Because this effect is only minor, however, it remains unknown whether these observations are physiologically significant. More recently, overexpression of FHL2/SLIM3 has been shown to result in a marked decrease in cellular SK1 activity and also attenuated the antiapoptotic effects of SK1 (46). Some other SK1-interacting proteins, including RPK118 (43), PECAM-1 (38), and aminoacylase 1 (42) also appear to alter SK1 localization when overexpressed, suggesting their possible involvement in the restriction of this enzyme to various cellular locations. Thus, it remains clear that although activation of SK1 can be mediated by phosphorylation (9), other mechanisms for the modulation of the activity of this enzyme by protein-protein interactions exist. This includes the novel mechanism described in this study involving the direct interaction and activation of SK1 by eEF1A.



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