Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

The CCT/TRiC chaperonin is required for maturation of sphingosine kinase 1

Julia R. Zebol[a,1], Niamh M. Hewitt[a,1], Paul A.B. Moretti[a], Helen E. Lynn[a], Julie A. Lake[b], Peng Li[b], Mathew A. Vadas[a,c,2], Binks W. Wattenberg[a,3], Stuart M. Pitson[a,d,*]

[a] Hanson Institute, Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia
[b] Infectious Diseases Laboratories, Institute for Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia
[c] School of Medicine, University of Adelaide, Adelaide, SA 5005, Australia
[d] School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA 5005, Australia

ARTICLE INFO

Article history:
Received 11 July 2008
Received in revised form 7 August 2008
Accepted 11 August 2008
Available online 14 August 2008

Keywords:
Chaperonin
CCT
TRiC
Protein folding
Sphingosine kinase

ABSTRACT

Sphingosine kinase 1 (SK1) catalyses the generation of sphingosine 1-phosphate (S1P), a bioactive phospholipid that influences a diverse range of cellular processes, including proliferation, survival, adhesion, migration, morphogenesis and differentiation. SK1 is controlled by various mechanisms, including transcriptional regulation, and post-translational activation by phosphorylation and protein–protein interactions which can regulate both the activity and localisation of this enzyme. To gain a better understanding of the regulatory mechanisms controlling SK1 activity and function we performed a yeast two-hybrid screen to identify SK1-interacting proteins. Using this approach we identified that SK1 interacts with subunit 7 (η) of cytosolic chaperonin CCT (chaperonin containing t-complex polypeptide, also called TRiC for TCP-1 ring complex), a hexadecameric chaperonin that binds unfolded polypeptides and mediates their folding and release in an ATP-dependent manner. Further analysis of the SK1–CCTη interaction demonstrated that other CCT/TRiC subunits also associated with SK1 in HEK293T cell lysates in an ATP-sensitive manner, suggesting that the intact, functional, multimeric CCT/TRiC complex associated with SK1. Furthermore, pulse-chase studies indicated that CCT/TRiC binds specifically to newly translated SK1. Finally, depletion of functional CCT/TRiC through the use of RNA interference in HeLa cells or temperature sensitive CCT yeast mutants reduced cellular SK1 activity. Thus, combined this data suggests that SK1 is a CCT/TRiC substrate, and that this chaperonin facilitates folding of newly translated SK1 into its mature active form.

1. Introduction

Sphingosine kinases (SKs) catalyse the generation of the bioactive phospholipid sphingosine 1-phosphate (S1P), which influences a diverse range of cellular processes, including proliferation, survival, adhesion, migration, morphogenesis and differentiation (Pébay et al., 2007; Hannun and Obeid, 2008). Many of the actions of S1P are mediated through S1P-specific G-protein-coupled receptors of which five have been identified. These cell-surface receptors are differentially expressed and linked to different G proteins, allowing S1P to elicit a variety of somewhat cell-specific responses through the activation of classic G_i, G_q and G_{12} signalling pathways (Rosen and Goetzl, 2005). In addition to this cell surface receptor-mediated signalling, S1P also appears to have intracellular second messenger functions, especially in calcium homeostasis, cell growth, and suppression of apoptosis (Spiegel and Milstien, 2003).

Cellular levels of S1P are largely regulated by SKs, of which two mammalian isoforms have been described (SK1 and SK2). Numerous studies have implicated SK1, but not SK2, in enhancing cell proliferation and survival, with forced overexpression of this enzyme also inducing NIH3T3 fibroblasts to acquire the transformed phenotype and the ability to form tumours in mice (Xia et al., 2000). More recently, evidence has emerged in support of a role for elevated cellular SK1 levels in the formation and/or progression of naturally occurring tumours, leading to considerable interest in SK1 as a target for anticancer therapies (Cuvillier, 2007).

SK1 is regulated by various mechanisms. A number of cellular stimuli can induce upregulation of SK1 expression, including estrogen (Sukocheva et al., 2003), epidermal growth factor (Döll et al.,

* Corresponding author at: Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia.
Tel.: +61 8 8222 3472; fax: +61 8 8232 4092.
E-mail address: stuart.pitson@imvs.sa.gov.au (S.M. Pitson).
[1] These authors contributed equally to the work.
[2] Present address: Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia.
[3] Present address: Departments of Medicine and Biochemistry and Molecular Biology, University of Louisville, Louisville, KY, USA.

1357-2725
doi:10.1016/j.biocel.2008.08.012

2005), transforming growth factor-β (Yamanaka et al., 2004) and histamine (Huwiler et al., 2006). Post-translational regulation of SK1 also occurs via phosphorylation at Ser225 (in human SK1). This phosphorylation not only directly enhances the catalytic activity of SK1, but is also required for its translocation from the cytoplasm to the plasma membrane which is important for its signalling functions (Pitson et al., 2003; Pitson et al., 2005). Furthermore, the activity and subcellular localisation of SK1 also appears to be regulated by a variety of interacting proteins, including eukaryotic elongation factor 1A (eEF1A) (Leclercq et al., 2008), TRAF2 (Xia et al., 2002), SK1-interacting protein (SKIP) (Lacana et al., 2002), PECAM-1 (Fukuda et al., 2004), and Lyn and Fyn tyrosine kinases (Olivera et al., 2006; Sun et al., 2006). The molecular mechanisms whereby these protein–protein interactions regulate SK1, however, are yet to be determined.

To gain a better understanding of 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 the cytosolic chaperonin CCT/TRiC (CCT for chaperonin containing t-complex polypeptide, but also called TRiC for TCP-1 ring complex) interacts with SK1. Furthermore, we show that CCT/TRiC binds specifically to newly translated SK1 and mediates its folding into its mature active form. Thus, this represents another level of regulation of cellular SK1 levels and activity.

2. Materials and methods

2.1. 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 (Pitson et al., 2000a) was cloned into pGBKT7 (Clontech) in-frame with the Gal4 DNA-binding domain. This bait construct was then transformed into *Saccharomyces cerevisiae* AH107 together with a human leukocyte cDNA library in pACT2 (Clontech). A total of 1×10^6 transformants were screened for growth on yeast synthetic dropout (SD) medium lacking tryptophan, leucine and histidine. Total plasmid DNA was then isolated from surviving His^+ colonies, pACT2 plasmids amplified in *Escherichia coli* JM109, and secondary screening for false positives performed by transformation of these isolated library plasmids into *S. cerevisiae* AH107 containing either pGBKT7-SK1 or empty pGBKT7. Confirmed positives where identified as those enabling yeast growth on SD medium lacking tryptophan, leucine and histidine only in the presence of pGBKT7-SK1, as well as enabling expression of β-galactosidase.

2.2. Generation of CCTη mammalian expression construct

The partial CCTη sequence isolated from the yeast two-hybrid screen was analysed via the NCBI database to design primers for PCR amplification of the full-length CCTη (Genbank accession number **AF026292**). CCTη was PCR amplified from human foreskin fibroblast cDNA using the primers, 5′-TAGAATTCCCATGATGCCCACACCAGT-3′ and 5′-TAGAATTCAGTGGGGGCGGCCACGAC-3′, digested with *Eco*RI and cloned into pCMV(HA) mammalian expression vector (Clontech). The orientation was determined by restriction analysis with sequencing verifying the integrity of the resultant CCTη cDNA containing a hemaglutinin (HA) epitope-tag at the N-terminus.

2.3. Cell culture and transfections

Human embryonic kidney cells (HEK293T) and human cervical cancer (HeLa) cells were cultured and transfected as described previously (Pitson et al., 2000b; Leclercq et al., 2008). Cells were routinely harvested in 50 mM Tris/HCl buffer (pH 7.4) containing 10% glycerol, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA and Complete™ protease inhibitors (Roche Diagnostics GMbH, Mannheim, Germany). Cell lysis was enhanced by six passages through a 26 gauge needle and the lysates then centrifuged at 13,000 *g* for 10 min at 4 °C to remove insoluble material. Protein concentrations of cell lysates were determined with Coomassie Brilliant Blue reagent (Sigma) using bovine serum albumin as standard.

2.4. CCTζ-1 knock-down by siRNA

siRNA knock-down of CCT was performed in HeLa cells by transfection with CCTζ-1 siRNA duplexes (siRNA-1, 5′-UUUA-GCUCUGCGCAGAGCGACUAUG-3′, and siRNA-2, 5′-UAUCCCAUA-CGCCUACUUCUGCUGC-3′) or control siRNA (Invitrogen) using HiPerFect (Qiagen). The cells were then cultured for two days, transfected again with the same siRNAs, and cultured for a further two days before harvesting and analysing for CCTζ-1 levels and sphingosine kinase activity.

2.5. Pull-down, immunoprecipitation and Western blot analyses

For pull-down analyses recombinant SK1 was generated and purified as a glutathione S-transferase (GST)-fusion protein in *E. coli* as previously described (Pitson et al., 2000a). This GST-SK1 (1 μg) was then bound to glutathione-Sepharose (Amersham), added to cell lysates and incubated at 4 °C for 1 h with constant agitation. CCTη associated with SK1 was then determined by immunoblot with anti-HA antibodies (Sigma) to detect HA epitope tagged CCTη. Lysates were probed with GST alone bound to glutathione-Sepharose as a control. For immunoprecipitation antibodies were added to cell 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 Pharmacia Biotech) for 1 h at 4 °C and washed with cold lysis buffer. Following SDS-PAGE and transfer of the proteins to nitrocellulose membranes, immunoblots were performed using M2 anti-FLAG antibody (Sigma) to detect FLAG epitope tagged SK1 or anti-HA antibodies to detect CCTη. Polyclonal goat anti-CCTζ-1 and rat anti-CCTα antibodies were from Santa Cruz Biotechnology and Stressgen (Victoria, Canada), respectively. The immunocomplexes were detected with HRP-conjugated anti-mouse, -rabbit, -rat, or -goat IgG (Pierce) using an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech).

2.6. Sphingosine kinase assays

Sphingosine kinase activity was determined using D-*erythro*-sphingosine (Biomol, Plymouth Meeting, PA) and [γ-^{32}P]ATP as substrates as described previously (Roberts et al., 2004). One unit (U) of activity is defined as 1 pmol of S1P formed per minute per mg of protein.

2.7. Pulse-chase analysis

HEK293T cells were transfected with either pCMV(HA)-CCTη alone, or co-transfected with pcDNA3-SK1(FLAG) (Pitson et al., 2000a). After 16 h the cells were incubated for 1 h in methionine-free DMEM containing 10% dialysed foetal calf serum, and then pulse labelled with fresh methionine-free DMEM containing 100 μCi/ml [^{35}S]methionine/cysteine (MP Biomedicals, CA) for 5 min. Cells were then washed and chased for 45 min in DMEM containing 10% foetal calf serum, 20 mM unlabelled methionine and

20 mM unlabelled cysteine. Extracts were prepared by lysing cells on ice in 25 mM Tris/HCl buffer (pH 7.4) containing 10% glycerol, 0.5% Nonidet P-40, 75 mM NaCl, 10 mM EDTA, 5 mM NaF, 5 mM β-glycerophosphate, 1 mM Na_3VO_4 and protease inhibitors. Cell lysis was enhanced by six passages through a 26 gauge needle and the lysates then centrifuged at 13,000 *g* for 10 min at 4 °C to remove insoluble material. Clarified cell lysates were then divided and immunoprecipitation of CCTη and SK1 achieved via their HA and FLAG epitopes, respectively, with immunocomplexes isolated using protein A-Sepharose. Co-immunoprecipitation of SK1 and CCTη in the immunocomplexes were then assessed by immunoblot analysis, and ^{35}S-incorporation into the immunoprecipitated proteins determined by Phosphorimaging of dried gels following SDS-PAGE.

2.8. Yeast CCT studies

A temperature-sensitive CCTδ (*anc2-1* mutant) strain of *S. cerevisiae* (DDY299) and its corresponding wildtype strain (DDY186) were kindly provided by David Drubin (University of California, Berkeley) (Vinh and Drubin, 1994). Human SK1 cDNA was transformed into these stains in pGBKT7 with transformants isolated on yeast SD plates lacking leucine. Cells were then grown in liquid SD medium lacking leucine at 25 °C, divided, and duplicate 30 ml cultures then growth at 25 °C and 35 °C (permissive and non-permissive mutant CCTδ temperatures, respectively) for 8 h. Cells were then harvested by centrifugation at 10,000 *g* for 5 min at 4 °C, resuspended in 50 mM Tris/HCl buffer (pH 7.4) containing 10% glycerol, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA and Complete™ protease inhibitors, and following addition of 0.3 g of glass beads (212-300 μm; Sigma) lysed by vortexing 3 × 1 min at 2500 rpm. Cell lysates were then centrifuged at 13,000 *g* for 10 min at 4 °C to remove insoluble material and assayed for total protein and sphingosine kinase activity.

3. Results and discussion

3.1. CCTη interacts with SK1

To gain a better understanding of the regulatory mechanisms controlling SK1 function, we performed a yeast two-hybrid screen to identify proteins that interact with SK1. Human SK1 was used as bait to screen a human leukocyte cDNA library. One partial cDNA isolated from this screen was of subunit 7 of the chaperonin CCT/TRiC complex (CCTη), corresponding to the c-terminal 200 amino acids of this 59 kDa protein (residues 344–543).

Since yeast two-hybrid screens can generate false positive results, putative protein–protein interactions identified by this methodology must be confirmed by other approaches. To do this full-length CCTη cDNA was amplified from human foreskin fibroblast cDNA and cloned into pCMV(HA) mammalian expression vector. The resultant HA epitope-tagged CCTη was then expressed in HEK293T cells, and its putative interaction with SK1 initially assessed via pull-down analysis using purified recombinant GST-SK1. Consistent with the yeast two-hybrid screen, these pull-down analyses showed an interaction between CCTη and SK1 since CCTη was isolated specifically with glutathione-Sepharose loaded with GST-SK1, but not with GST alone (Fig. 1A). The interaction between SK1 and CCTη was then further confirmed by co-immunoprecipitation analyses using lysates from HEK293T cells co-expressing HA-tagged CCTη and FLAG-tagged SK1 (Fig. 1B). Consistent with the results of the pull-down experiments, the presence of SK1 in the anti-HA (CCTη) immunocomplexes was observed. Together, this data provided considerable evidence supporting the existence of an interaction between SK1 and CCTη in mammalian cells.

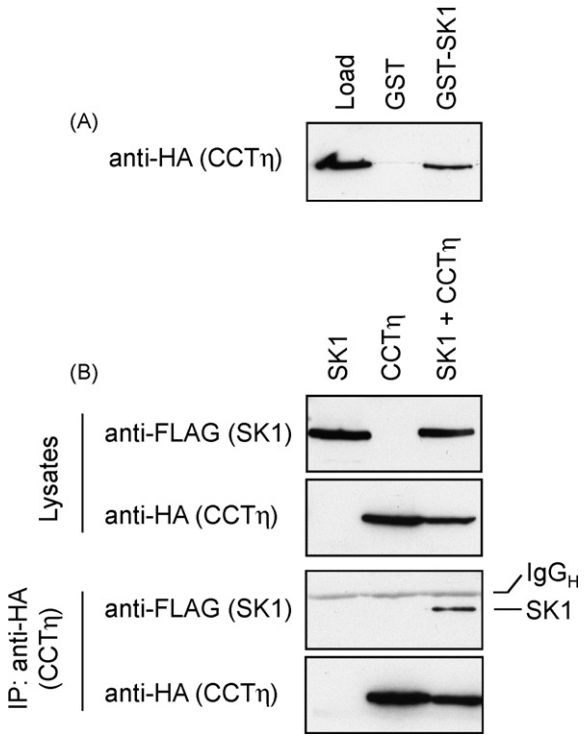

Fig. 1. CCTη interacts with SK1. (A) GST-SK1 bound to GSH-Sepharose was incubated with lysates from HEK293T cells expressing HA-tagged CCTη. CCTη pulled down by the GST-SK1 was detected by immunoblot with anti-HA antibodies. (B) Lysates from HEK293T cells co-transfected with FLAG-tagged SK1 and HA-tagged CCTη were immunoprecipitated (IP) with anti-HA and SK1 present in the immunocomplexes detected by immunoblot with anti-FLAG antibodies. IgG_H represents the heavy chain of the anti-HA antibody. Results are representative of three independent experiments.

3.2. CCTη overexpression has no effect on cellular SK1 activity or activation

A previous study (Hanafy et al., 2004) suggested that CCTη could interact with soluble guanylyl cyclase and inhibit its nitric oxide-dependent activation both *in vitro* and when overexpressed in cells. Since SK1 is also an enzyme that can be rapidly and transiently activated (Pitson et al., 2003), and its catalytic activity can also be affected by interaction with a number of different proteins (Lacana et al., 2002; Fukuda et al., 2004; Fujita et al., 2004; Maceyka et al., 2004; Urtz et al., 2004; Olivera et al., 2006; Sun et al., 2006; Leclercq et al., 2008), we examined the possible effects of CCTη overexpression on basal cellular SK1 activity and its agonist-dependent activation. Unlike the situation with soluble guanylyl cyclase, however, overexpression of CCTη in HEK293T cells had no detectable effect on either basal SK1 activity, or SK1 activation induced by cell treatment with phorbol esters or tumour necrosis factor-α (Fig. 2), two agonists previously shown to activate this enzyme (Pitson et al., 2000b).

3.3. CCT acts as a SK1 chaperonin

CCTη is one subunit of the CCT/TRiC complex, a large double ring-shaped, hexadecameric chaperonin composed of eight different subunits (Valpuesta et al., 2002). The CCT/TRiC chaperonin binds unfolded polypeptides in its central cavity and mediates folding and release in a Mg^{2+}/ATP-dependent manner (Frydman,

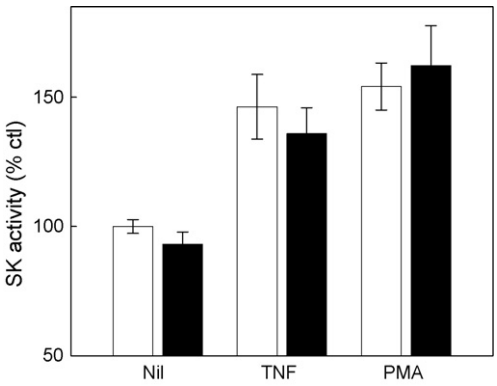

Fig. 2. CCTη overexpression has no effect on cellular SK1 activity or activation. HEK293T cells overexpressing CCTη (filled bars) were left untreated (Nil), or exposed to TNFα (1 ng/ml) for 10 min or PMA (100 ng/ml) for 30 min and then harvested and analysed for endogenous SK activity, and compared to empty vector transfected cells (open bars). Data is mean ± S.D. of three independent experiments.

2001). To investigate the possible interaction of the intact CCT/TRiC complex with SK1 we initially examined the presence of other CCT/TRiC subunits in pull-down analyses with GST-SK1 from lysates of untransfected HEK293T cells. In addition to CCTη, both CCTα and CCTη subunits were also found to specifically associate with SK1 (Fig. 3A) suggesting that these observed interactions may be as a result of the intact chaperonin CCT/TRiC complex associating with SK1. To examine this possibility further we examined the Mg^{2+}/ATP dependence of the interaction between CCTη and SK1 since the action and subsequent dissociation of functional chaperonins from their substrates has an absolute requirement on this nucleotide (Spiess et al., 2004). Consistent with the potential role

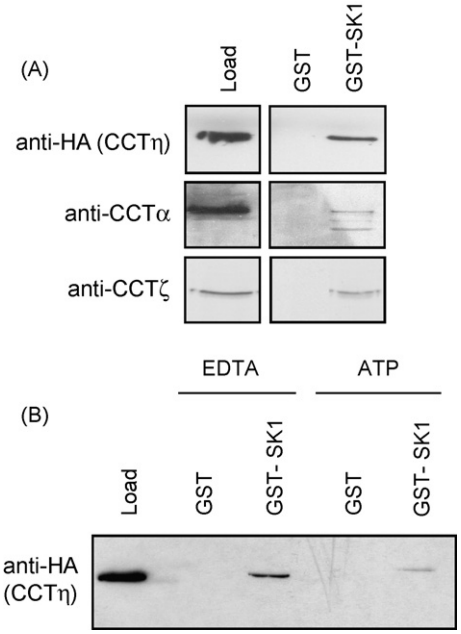

Fig. 3. The TRiC/CCT chaperonin complex interacts with SK1. (A) GST-SK1 bound to GSH-Sepharose was incubated with lysates from HEK293T cells expressing HA-tagged CCTη. The presence of TRiC/CCT subunits in these GST-SK1 pull-downs were detected by immunoblotting with anti-CCTα, anti-CCTζ and anti-HA (for CCTη) antibodies. (B) The ATP-dependence of the SK1–CCTη interaction was assessed using lysates from HEK293T cells transfected with HA-tagged CCTη that were subjected to the same pull-down analysis, but performed in the presence of either 5 mM ATP or 5 mM EDTA to chelate Mg^{2+} and thus reduce available Mg^{2+}/ATP. Results are representative of more than three independent experiments.

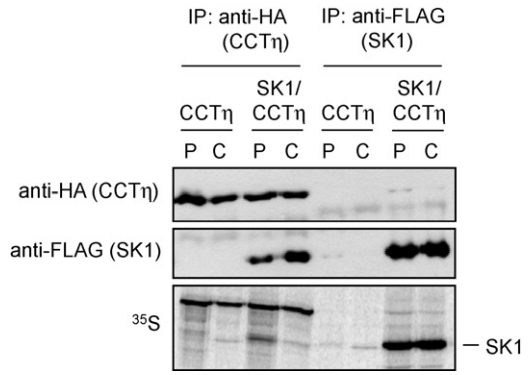

Fig. 4. TRiC/CCT associates with newly translated SK1. HEK293T cells co-transfected with FLAG-tagged SK1 and HA-tagged CCTη were pulse-labelled with [35S]methionine/cysteine for 5 min (P), chased for 45 min (C), then harvested, lysed and immunoprecipitated with either anti-HA (for CCTη) or anti-FLAG (for SK1) antibodies. Phosphorimager analysis of the immune complexes after SDS-PAGE showed the presence of 35S-labelled proteins in the CCTη immunoprecipitates during the pulse and not chase phases indicating that TRiC/CCT associates with newly translated SK1. The presence of CCTη and SK1 in the immune complexes was confirmed by immunoblot analysis, while anti-FLAG immunoprecipitations demonstrated that equal 35S-labelled SK1 was present in the cells harvested after the pulse and chase phases. Results are representative of three independent experiments.

of CCT/TRiC as a SK1 chaperonin, the association of CCTη with SK1 was found to be markedly decreased in the presence of Mg^{2+}/ATP (Fig. 3B).

3.4. CCT interacts with newly synthesized SK1 and is necessary for SK1 maturation

The CCT/TRiC chaperonin appears to have a primary cellular function of folding some newly translated polypeptides. While tubulin and actin folding appear to constitute the predominant activity of CCT/TRiC (Siegers et al., 2003), this chaperonin is also involved in the folding of a distinct set of other cellular, non-cytoskeletal proteins (Thulasiraman et al., 1999; Camasses et al., 2003; Spiess et al., 2004; Dekker et al., 2008). To determine whether CCT/TRiC may be required for SK1 folding, we initially examined the timing of CCTη interaction with SK1 in a pulse chase experiment (Fig. 4). Newly translated SK1 was found associated with CCTη but was released during the subsequent chase, consistent with a possible role of CCT/TRiC in folding of newly translated SK1.

The CCT/TRiC chaperonin is highly conserved amongst eukaryotic species from yeast to humans (Valpuesta et al., 2002). Thus, the yeast *S. cerevisiae*, where a mutant possessing a temperature sensitive variant of CCT4 (CCTδ) that inactivates the function of the yeast CCT chaperonin has been characterised (Vinh and Drubin, 1994; Shimon et al., 2008), provided a useful system to initially examine the role of CCT/TRiC in folding and maturation of SK1. Human SK1 was expressed in both wildtype and the temperature sensitive CCT mutant of *S. cerevisiae*, the yeast grown at the permissive and non-permissive temperatures (25 °C and 35 °C, respectively) for 8 h, harvested and sphingosine kinase activity in the yeast cell lysates determined (Fig. 5). Consistent with a role of CCT in SK1 maturation, ablation of yeast CCT function by incubation of the temperature sensitive CCT mutant at the non-permissive temperature lead to a substantial decrease in SK1 activity that was not observed in the wildtype yeast.

To further examine the role of the CCT/TRiC chaperonin in SK1 folding and maturation in human cells we employed a siRNA approach to knock-down levels of CCTζ-1. This, in turn, has been previously shown to decrease the cellular levels of the other members of the CCT/TRiC complex and attenuate folding and function

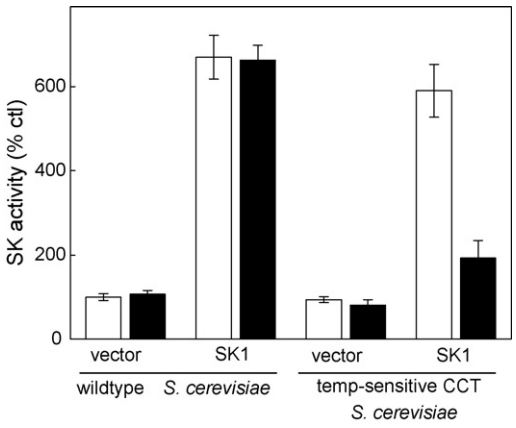

Fig. 5. Use of temperature-sensitive CCTδ mutant yeast indicates a role for CCT in SK1 function. Human SK1 was constitutively expressed in a temperature-sensitive CCTδ strain of *S. cerevisiae* and its corresponding wildtype strain. Control yeast strains were transduced with the empty vector. The yeast cultures were initially grown at 25 °C, and then incubated for 8 h at 25 °C (open bars) and 35 °C (filled bars) which represented the permissive and non-permissive mutant CCTδ temperatures, respectively. Cells were then harvested, lysed and assayed for total SK activity. SK activity in vector control cells represents endogenous yeast SK.

of substrate proteins (Kunisawa and Shastri, 2003; Grantham et al., 2006). Knock-down of CCTζ-1 in HeLa cells lead to a substantial decrease in cellular sphingosine kinase activity (Fig. 6). Although these cells still retained some active SK1, possibly due to both the presence of residual CCT/TRiC and the stability of SK1 (Kihara et al., 2006), these results were, again, consistent with an important role of CCT/TRiC in SK1 maturation.

3.5. Conclusions and implications of this study

A substantial fraction of newly synthesized polypeptides appear to fold spontaneously in the cell (Frydman, 2001). Folding of the remaining proteins is achieved via molecular chaperones, of which two main systems exist in eukaryotes: the heat shock protein (hsp) 70/hsp40 system, and CCT/TRiC chaperonin (Frydman, 2001). In this study we have presented data that supports an essential role for the CCT/TRiC chaperonin in folding and maturation of newly translated SK1.

A number of CCT/TRiC substrates have been identified (Ho et al., 2002; Gavin et al., 2002; Spiess et al., 2004; Dekker et al., 2008), including cytoskeletal proteins such as actin and tubulin, and signalling proteins such as Cdc20 (Camasses et al., 2003), cyclin E (Won et al., 1998), polo-like kinase 1 (Plk1) (Liu et al., 2005), HIF prolyl hydroxylase PHD3 (Masson et al., 2004), G_{α}-transducin (Farr et al., 1997), and von Hippel-Lindau tumour suppressor protein (VHL) (Feldman et al., 1999). Indeed, CCT/TRiC appears to be involved in maturation of a diverse but distinct subset of cellular proteins, which although estimated in one study to be up to 15% of newly synthesised polypeptides (Thulasiraman et al., 1999), this figure included the highly abundant substrates actin and tubulin which may consume 50–60% of cellular CCT/TRiC activity (Siegers et al., 2003). Most CCT/TRiC substrates appear to be between 30 and 60 kDa (Thulasiraman et al., 1999), and be either prone to aggregation or components of oligomeric complexes (Dunn et al., 2001; Spiess et al., 2004). In this light, SK1 could be considered a typical CCT/TRiC substrate since it is an aggregation-prone 43 kDa protein (Pitson et al., 2000a). Indeed, we have observed that diverse point mutations and short deletions render SK1 unfolded and catalytically inactive (Pitson et al., 2002). Once folded, however, SK1 is relatively stable to heat and pH (Pitson et al., 2000a). Notably, SK1 interacts with membranes (Pitson et al., 2005), and with a hydrophobic substrate. The sensitivity of SK1 to folding perturbations, and the participation of CCT/TRiC, may reflect a folding pathway that must avoid aberrant interactions between hydrophobic domains. In addition, while catalytically active as a monomer, SK1 is known to form stable complexes in cells with a number of other proteins that appear to regulate its catalytic activity and cellular localisation (Olivera et al., 2006; Sun et al., 2006; Leclercq et al., 2008). Notably, CCT/TRiC has been proposed to play a role in the formation of functional multiprotein complexes, including the VHL-elongin B/C complex (Feldman et al., 1999), cdc20-anaphase promoting complex (Camasses et al., 2003), and G protein βγ-dimer formation (Wells et al., 2006). Thus, it is tempting to speculate that CCT/TRiC may play a role in the regulation of SK1 via facilitating interaction with regulatory proteins such as eEF1A (Leclercq et al., 2008), TRAF2 (Xia et al., 2002) or Lyn and Fyn tyrosine kinases (Olivera et al., 2006; Sun et al., 2006).

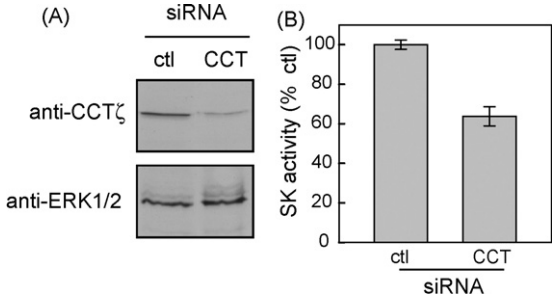

Fig. 6. siRNA-mediated knock-down of CCTζ decreases endogenous SK1 activity. (A) siRNAs directed against CCTζ-1 were transfected into HeLa cells and the decrease in the overall level of CCTζ-1 protein determined in the cell lysates by immunoblot with anti-CCTζ antibodies. Immunoblots for ERK1/2 were used as loading controls. Densitometry revealed an approximate 70% knockdown in CCTζ-1 levels. (B) Lysates from HeLa cells transfected with CCTζ-1 siRNA (CCT siRNA) or control siRNA (ctl siRNA) were analysed for endogenous SK activity. Data is mean ± S.D. for three independent experiments. Data shown is for CCTζ-1 siRNA-1, with similar results observed for siRNA-2 (data not shown).

In conclusion, the data presented in this study suggests that SK1 is a CCT/TRiC substrate. This chaperonin appears to facilitate folding of newly translated SK1 into its mature active form, which represents another level of regulation of cellular SK1 levels and activity.

Acknowledgements

This work was supported by the Fay Fuller Foundation and a Senior Research Fellowship (508098) from the National Health and Medical Research Council of Australia to S.M.P.

References

Camasses A, Bogdanova A, Shevchenko A, Zachariae W. The CCT chaperonin promotes activation of the anaphase-promoting complex through the generation of functional Cdc20. Mol Cell 2003;12:87–100.
Cuvillier O. Sphingosine kinase-1: a potential therapeutic target in cancer. Anticancer Drugs 2007;18:105–10.
Dekker C, Stirling PC, McCormack EA, Filmore H, Paul A, Brost RL, et al. The interaction network of the chaperonin CCT. EMBO J 2008;27:1827–39.
Döll F, Pfeilschifter J, Huwiler A. The epidermal growth factor stimulates sphingosine kinase-1 expression and activity in the human mammary carcinoma cell line MCF7. Biochim Biophys Acta 2005;1738:72–81.
Dunn AY, Melville MW, Frydman J. Cellular substrates of the eukaryotic chaperonin TRiC/CCT. J Struct Biol 2001;135:176–84.
Farr GW, Scharl EC, Schumacher RJ, Sondek S, Horwich AL. Chaperonin-mediated folding in the eukaryotic cytosol proceeds through rounds of release of native and nonnative forms. Cell 1997;89:927–37.
Feldman DE, Thulasiraman V, Ferreyra RG, Frydman J. Formation of the VHL-elongin BC tumor suppressor complex is mediated by the chaperonin TRiC. Mol Cell 1999;4:1051–61.

Frydman J. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu Rev Biochem 2001;70:603–47.
Fujita T, Okada T, Hayashi S, Jahangeer S, Miwa N, Nakamura S. δ-catenin/NPRAP (neural plakophilin-related armadillo repeat protein) interacts with and activates sphingosine kinase 1. Biochem J 2004;382:717–23.
Fukuda Y, Aoyama Y, Wada A, Igarashi Y. Identification of PECAM-1 association with sphingosine kinase 1 and its regulation by agonist-induced phosphorylation. Biochim Biophys Acta 2004;1636:12–21.
Gavin AC, Bösche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 2002;415:141–7.
Grantham J, Brackley KI, Willison KR, Substantial. CCT activity is required for cell cycle progression and cytoskeletal organisation in mammalian cells. Exp Cell Res 2006;312:2309–24.
Hanafy KA, Martin E, Murad F. CCTη, a novel soluble guanylyl cyclase-interacting protein. J Biol Chem 2004;279:46946–53.
Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol 2008;9:139–50.
Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, et al. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature 2002;415:180–3.
Huwiler A, Döll F, Ren S, Klawitter S, Greening A, Römer I, et al. Histamine increases sphingosine kinase-1 expression and activity in the human arterial endothelial cell line EA.hy 926 by a PKC-α-dependent mechanism. Biochim Biophys Acta 2006;1761:367–76.
Kihara A, Anada Y, Igarashi Y. Mouse sphingosine kinase isoforms SPHK1a and SPHK1b differ in enzymatic traits including stability, localization, modification, and oligomerization. J Biol Chem 2006;281:4532–9.
Kunisawa J, Shastri N. The group II chaperonin TRiC protects proteolytic intermediates from degradation in the MHC class I antigen processing pathway. Mol Cell 2003;12:565–76.
Lacana E, Maceyka M, Milstien S, Spiegel S. Cloning and characterization of a protein kinase A anchoring protein (AKAP)-related protein that interacts with and regulates sphingosine kinase 1 activity. J Biol Chem 2002;277:32947–53.
Leclercq TM, Moretti PAB, Vadas MA, Pitson SM. Eukaryotic elongation factor 1A interacts with sphingosine kinase and directly enhances its catalytic activity. J Biol Chem 2008;283:9606–14.
Liu X, Lin CY, Lei M, Yan S, Zhou T, Erikson RL. CCT chaperonin complex is required for the biogenesis of functional Plk1. Mol Cell Biol 2005;25:4993–5010.
Maceyka M, Nava VE, Milstien S, Spiegel S. Aminoacylase 1 is a sphingosine kinase 1-interacting protein. FEBS Lett 2004;568:30–4.
Masson N, Appelhoff RJ, Tuckerman JR, Tian YM, Demol H, Puype M, et al. The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin. FEBS Lett 2004;570:166–70.
Olivera A, Urtz N, Mizugishi K, Yamashita Y, Gilfillan AM, Furumoto Y, et al. IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses. J Biol Chem 2006;281:2515–25.
Pébay A, Bonder CS, Pitson SM. Stem cell regulation by lysophospholipids. Prostoglandins Other Lipid Mediat 2007;84:83–97.
Pitson SM, D'Andrea RJ, Vandeleur L, Moretti PAB, Xia P, Gamble JR, et al. Human sphingosine kinase: purification, molecular cloning and characterisation of the native and recombinant enzymes. Biochem J 2000a;350:429–41.
Pitson SM, Moretti PAB, Zebol JR, Xia P, Gamble JR, Vadas MA, et al. Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation: a dominant-negative sphingosine kinase. J Biol Chem 2000b;275:33945–50.
Pitson SM, Moretti PAB, Zebol JR, Zareie R, Derian CK, Darrow AL, et al. The nucleotide-binding site of human sphingosine kinase 1. J Biol Chem 2002;277:49545–53.
Pitson SM, Moretti PAB, Zebol JR, Lynn HE, Xia P, Vadas MA, et al. Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J 2003;22:5491–500.
Pitson SM, Xia P, Leclercq TM, Moretti PAB, Zebol JR, Lynn HE, et al. Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. J Exp Med 2005;201:49–54.
Roberts JL, Moretti PAB, Darrow AL, Derian CK, Vadas MA, Pitson SM. An assay for sphingosine kinase activity using biotinylated sphingosine and streptavidin-coated membranes. Anal Biochem 2004;331:122–9.
Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: signaling and biology. Nat Rev Immunol 2005;5:560–70.
Shimon L, Hynes GM, McCormack EA, Willison KR, Horovitz A. ATP-induced allostery in the eukaryotic chaperonin CCT is abolished by the mutation G345D in CCT4 that renders yeast temperature-sensitive for growth. J Mol Biol 2008;377:469–77.
Siegers K, Bölter B, Schwarz JP, Böttcher UM, Guha S, Hartl FU. TRiC/CCT cooperates with different upstream chaperones in the folding of distinct protein classes. EMBO J 2003;22:5230–40.
Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol 2003;4:397–407.
Spiess C, Meyer AS, Reissmann S, Frydman J. Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. Trends Cell Biol 2004;14:598–604.
Sukocheva OA, Wang L, Albanese N, Pitson SM, Vadas MA, Xia P. Sphingosine kinase transmits estrogen signaling in human breast cancer cells. Mol Endocrinol 2003;17:2002–12.
Sun J, Yan G, Ren A, You B, Lia JK. FHL2/SLIM3 decreases cardiomyocyte survival by inhibitory interaction with sphingosine kinase-1. Circ Res 2006;99:468–76.
Thulasiraman V, Yang CF, Frydman J. In vivo newly translated polypeptides are sequestered in a protected folding environment. EMBO J 1999;18:85–95.
Urtz N, Olivera A, Bofill-Cardona E, Csonga R, Billich A, Mechtcheriakova D, et al. Early activation of sphingosine kinase in mast cells and recruitment to Fc(RI are mediated by its interaction with Lyn kinase. Mol Cell Biol 2004;24:8765–77.
Valpuesta JM, Martin-Benito J, Gómez-Puertas P, Carrascosa JL, Willison KR. Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. FEBS Lett 2002;529:11–6.
Vinh DB, Drubin DG. A yeast TCP-1-like protein is required for actin function in vivo. Proc Natl Acad Sci U S A 1994;91:9116–20.
Wells CA, Dingus J, Hildebrandt JD. Role of the chaperonin CCT/TRiC complex in G protein βγ-dimer assembly. J Biol Chem 2006;281:20221–32.
Won KA, Schumacher RJ, Farr GW, Horwich AL, Reed SI. Maturation of human cyclin E requires the function of eukaryotic chaperonin CCT. Mol Cell Biol 1998;18:7584–9.
Xia P, Gamble JR, Wang L, Pitson SM, Moretti PAB, D'Andrea RJ, et al. An oncogenic role of sphingosine kinase. Curr Biol 2000;10:1527–30.
Xia P, Wang L, Moretti PAB, Albanese N, Chai F, Pitson SM, et al. Sphingosine kinase interacts with TRAF2 and dissects TNF signalling. J Biol Chem 2002;277:7996–8003.
Yamanaka M, Shegogue D, Pei H, Bu S, Bielawska A, Bielawski J, et al. Sphingosine kinase 1 (SPHK1) is induced by transforming growth factor-β and mediates TIMP-1 up-regulation. J Biol Chem 2004;279:53994–4001.