Chaperonin containing TCP-1 (CCT) is a large multisubunit complex that mediates protein folding in eukaryotic cells. CCT participates in the folding of newly synthesized polypeptides, including actin, tubulin, and several cell cycle regulators; therefore, CCT plays an important role in cytoskeletal organization and cell division. Here we identify the chaperonin CCT as a novel physiological substrate for p90 ribosomal S6 kinase (RSK) and p70 ribosomal S6 kinase (S6K). RSK phosphorylates the β subunit of CCT in response to tumor promoters or growth factors that activate the Ras-mitogen-activated protein kinase (MAPK) pathway. CCTβ Ser-260 was identified as the RSK site by mass spectrometry and confirmed by site-directed mutagenesis. RSK-dependent Ser-260 phosphorylation was sensitive to the MEK inhibitor U0126 and the RSK inhibitor BID-1870. Insulin weakly activates RSK but strongly activates the phosphoinositide 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway and utilizes S6K to regulate CCTβ phosphorylation. Thus, the Ras-PI3K and PI3K-mTOR pathways converge on CCTβ Ser-260 phosphorylation in response to multiple agonists in various mammalian cells. We also show that RNA interference-mediated knockdown of endogenous CCTβ causes impaired cell proliferation that can be rescued with ectopically expressed murine CCTβ wild-type or phosphomimetic mutant S260D, but not the phosphorylation-deficient mutant S260A. Although the molecular mechanism of CCTβ regulation remains unclear, our findings demonstrate a link between oncogene and growth factor signaling and chaperonin CCT-mediated cellular activities.

The molecular mass ~90-kDa ribosomal S6 kinases (RSK) and molecular mass ~70-kDa ribosomal S6 kinases (S6K) are distinct families of Ser/Thr kinases that regulate diverse cellular processes. RSK is activated by extracellular-signal-regulated kinase (ERK) in the Ras-mitogen-activated protein kinase (MAPK) pathway (1, 2). RSK phosphorylates a variety of proteins, including transcription factors, immediate-early gene products, translational regulators, enzymes, and structural proteins, that potentially link it to many biological processes such as cell proliferation, cell differentiation, and survival (3). S6K acts as a downstream mediator of mammalian target of rapamycin (mTOR) in the phosphoinositide 3-kinase (PI3K) pathway and/or the Ras-MAPK pathway, and regulates cell growth. A number of S6K substrates identified so far include factors involved in the regulation of mRNA translation, highlighting an important role of S6k in protein synthesis (4). Recent studies have revealed that RSK and S6K collaboratively regulate various biological processes, including translational control.

Translational control is modulated by various extracellular stimuli. Signaling pathways regulate efficient assembly of components of the translational machinery and also stimulate ribosome biogenesis to facilitate efficient protein synthesis (5–7). The PI3K-mTOR pathway plays a critical role in this process, whereas the Ras-PI3K pathway converges at various common as well as unique points and therefore also modulates translational activity in cells. RSK or Akt phosphorylation of TSC2 at unique and overlapping sites results in activation of mTOR-S6K pathway leading to translation initiation (8, 9). RSK-mediated raport phosphorylation also enhances mTOR kinase activity (10). RSK and S6K phosphorylate eukaryotic initiation factor 4B at Ser-422, which is important for its recruitment into the translation preinitiation complex (11–13). S6K phosphorylates the 40 S ribosomal protein S6 at Ser-235, Ser-236, Ser-240, Ser-244, and Ser-247, where RSK phosphorylation of the ribosomal S6 protein at Ser-235/236 also correlates with induction of cap-dependent translation (14). Thus, S6K and RSK are regarded as critical regulators for growth factor-mediated translational control.

The identification and functional characterization of novel substrates for RSK and S6K is essential for expanding our understanding of the physiological function of two different families of ribosomal S6 kinases in cells. To achieve this, we have applied proteomic approaches. A unique antibody raised against the consensus Akt phosphorylation motif RXRXXpS/pT has been successfully used to identify Akt substrates, including Tuberin (8), PRAS40 (15), AS160 (16), p122RhoGAP (17), and peripherin (18). Based on the fact that RSK and S6K belong to the AGC (protein kinases A, G, and C)
Phosphorylation of CCT by RSK and S6K

Kinase superfamily, which display a preference for basophilic sites, including the RXRXXpS/pT motif, we used this antibody to identify additional substrates for RSK and S6K.

Here we report the identification of the eukaryotic chaperonin containing TCP-1 (CCT) as a downstream target for RSK and S6K in the Ras-MAPK and PI3K-mTOR pathways. The chaperonin CCT, also known as TRiC, is composed of eight different subunits and is the protein folding machinery that binds nascent polypeptides from ribosomes (19–21). Although initially proposed to fold only actin and tubulin (22–25), an increasing number of physiological CCT substrates have been identified; these include cyclin E, cdc20, polo-like kinase 1, and Von Hippel-Lindau tumor suppressor protein (26–28). Some data indicate that ~5–10% of newly synthesized proteins may flow through CCT (29, 30). Given the connection between mitogen-stimulated kinases and enhanced protein synthetic rates, we hypothesized that the protein folding activity of CCT might provide a mechanism to coordinate S6K- and RSK-regulated protein synthesis with protein folding, and in so doing, also reduce potential unfolded protein stress responses. As a first step in addressing this potential link between growth factor signaling and the biological processes regulated by CCT, we show that RSK and S6K phosphorylate the CCTβ subunit at Ser-260. Furthermore, we show that CCTβ plays an important role in regulating cell proliferation and that Ser-260 phosphorylation contributes significantly to this process. Thus, the Ras-MAPK and PI3K-mTOR pathways utilize RSK and S6K to converge upon the phosphorylation and regulation of CCTβ function in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Anti-CCTα, anti-CCTβ, anti-CCTγ, and anti-actin antibodies were obtained from Santa Cruz Biotechnology. Anti-phospho-ERK1/2 antibody, anti-FLAG antibody, anti-FLAG M2 Affinity Gel, FLAG peptide, insulin, phorbol myristate acetate (PMA), epidermal growth factor (EGF), and Polybrene were purchased from Sigma. Anti-phospho-Akt substrate (αPAS) antibody was obtained from Cell Signaling Technology. Anti-RSK antibody was kindly provided from Zymed Laboratories Inc. Anti-ERK1/2 antibody was prepared in the laboratory (31). Anti-HA monoclonal antibodies were kindly provided by Margaret Chou (University of Pennsylvania). LY294002 and UO126 were purchased from Calbiochem.

Lipofectamine 2000 was purchased from Invitrogen. BI-D1870 was synthesized as described previously (32). Characterization of the synthesized compound by 1H NMR and reversed-phase liquid chromatography mass-spectrometry (LC-MS) confirmed a chemical structure consistent with BI-D1870.

Plasmids—The plasmids encoding HA-tagged human RSK1, mouse RSK2, and their kinase inactive mutants were used in this study. The human CCTβ cDNA was cloned into pKFLAG in fusion with a FLAG tag. Point mutated cDNA was generated by QuikChange site-directed mutagenesis (Stratagene), and fragments were cloned into pKFLAG, pGEX, and pLNCX2, respectively. The generation of pRK7-HA-WT S6K1 or Akt has been described previously (33).

Cell Culture, Transfection, and Viral Infection—HEK293E cells, U2-OS cells, and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. MCF10A cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 media containing 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin. Human mammary epithelial cells (HMECs) were obtained from Cambrex and grown in Dulbecco’s modified Eagle’s medium/F-12 media containing 10 ng/ml EGF, 10 μg/ml insulin, and 0.5 μg/ml hydrocortisone. The HMECs were immortalized by sequential infection with pBabeZevo-hTERT and pBabeHygro-dominant negative p53DD retrovirus (34). They were made to overexpress EGFR by infection with pzw1Blast-EGFR retrovirus and selection with 0.25 μg/ml Blastocidin. For transfection of small interference RNA (siRNA) or DNA plasmids, conventional calcium phosphate precipitation was performed. RSK siRNA was described elsewhere (35). Cells were grown for 24 h following transfection, starved for 24 h, and used for the assay. For short hairpin RNA (shRNA)-mediated knockdown of endogenous CCTβ, lentivirus was produced using the pLKO vector system (Open Biosystems), and cells infected in the presence of 8 μg/ml Polybrene. Two days after viral infection, cells were treated and selected with 2 μg/ml puromycin. shRNAs were obtained from Open Biosystems (shRNA1, TRCN0000029499; shRNA2, TRCN0000029450). Retrovirus was produced using pLNCX2 vector system to overexpress ectopic human or murine CCTβ, and 2 days after viral infection, cells were selected with 100 or 400 μg/ml G418 for HMECs or U2-OS cells, respectively.

Immunoprecipitation—Cells were extracted with lysis buffer A (10 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 1 mg/ml pepstatin) containing 0.2% Nonidet P-40. After centrifugation, supernatants were collected and added to the extracts. Samples were boiled for 5 min and electrophoresed by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (29). Cells were grown for 24 h following transfection, starved for 24 h, and used for the assay.

Immunoblot Analysis—For immunoblot analysis, the cells were extracted in the lysis buffer A, and reducing buffer was added to the extracts. Samples were boiled for 5 min and electrophoresed by SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Whatman). The membranes were blocked with TBST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 3% nonfat dried milk or 2% bovine serum albumin, and probed overnight with primary antibodies, washed three times for 10 min each, and then probed for 1 h with secondary antibodies coupled to peroxidase. Immunoblots were developed using enhanced chemiluminescence.

Cell Proliferation Assay—HMECs or U2-OS were seeded at 2 × 10⁴ cells per 6-cm plates and cultured with the indicated growth medium. The cells were harvested after trypsinization, and the number of cells was counted using a Beckman Z2 couler counter.

Column Chromatography—For Protocol 1, cells were extracted with lysis buffer B (10 mM Tris-HCl, pH 6.2, 50 mM
NaCl, 10 mM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 1 mg/ml pepstatin). After centrifugation, supernatants were filtered with a 0.45-μm filter unit and subjected to phenyl-Sepharose 6 Fast Flow chromatography (Amersham Biosciences). The fractionated samples were subjected to SP-Sepharose Fast Flow column chromatography as described above.

Mass Spectrometry—For identification of p54, the silver-stained band on the SDS-PAGE gel was excised, and the gel piece was destained as described before (36). The proteins in the gel were reduced, alkylated with iodoacetamide, and digested with trypsin. The resultant peptides were extracted, desalted with StageTip (37), and subjected to reversed-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a high resolution hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific) with TOP10 method, as described previously (38). The obtained data were searched against the IPI mouse data base (39) using the SEQUEST algorithm (40). Proteins were identified with at least two unique valid peptides, and the false discovery rate was estimated to be 0% using the target-decoy approach (41).

For identification of CCTβ phosphorylation sites, overexpressed and immunoprecipitated FLAG-CCTβ was subjected to SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. The CCTβ in the gel was destained, reduced, alkylated with iodoacetamide, and digested with trypsin, chymotrypsin, or lysyl endopeptidase (Lys-C). The resultant peptides were extracted, desalted, and subjected to LC-MS/MS analysis using both collision-induced dissociation and electron transfer dissociation. The obtained data were searched against FLAG-CCTβ sequence using SEQUEST. All candidate MS/MS spectra were manually inspected, and site localization reliability was assessed by Ascore (42).

**RESULTS**

Identification of the Chaperonin CCT as a Downstream Target for RSK in the Ras-MAPK Pathway—To search for novel targets of RSK and S6K, we used the Phospho-Akt substrate

FIGURE 1. The chaperonin CCT is a candidate substrate of RSK in the Ras-MAPK pathway. A: left; HEK293E cells were transfected with either the vector or RSK plasmids, serum-starved 24 h after transfection, and treated with DMSO or PMA for 15 min. Cell lysates were immunoblotted with anti-Phospho-Akt substrate antibody (αPAS), anti-RSK1, anti-RSK2, and anti-actin antibodies. Right; HEK293E cells were transfected with either the mock or RSK siRNA. Cells were treated as described above. B, fractionated samples from SP-Sepharose chromatography was loaded onto SDS-PAGE gel, and the gel was subjected to silver staining and mass spectrometry. C, HEK293E cells were serum-starved for 24 h and stimulated with DMSO or PMA for 15 min. Endogenous CCTβ was incubated with anti-CCTβ antibody for 2 h and immunoprecipitated. Cell lysates and immunoprecipitates were immunoblotted with αPAS and αCCTβ.
Phosphorylation of CCT by RSK and S6K

To confirm that RSK phosphorylates CCT, we conducted in vitro kinase assays. Incorporation of radioactive phosphate from \( \gamma^{32}P \)ATP was seen in the sample containing both HA-tagged RSK1 and the purified FLAG-tagged CCTβ protein. In a similar experiment without radioactive ATP, CCTβ phosphorylation was confirmed by immunoblotting with αPAS (Fig. 2D). Taken together, our findings indicate that RSK phosphorylates CCTβ in vitro and is the major kinase responsible for CCTβ phosphorylation in EGF- or PMA-stimulated HEK293E cells.

**CCTβ Ser-260 Is the Phosphorylation Site Recognized by αPAS**—To identify potential RSK phosphorylation sites, the immunoprecipitated FLAG-CCTβ was digested in-gel with trypsin, chymotrypsin, or Lys-C and subjected to LC-MS/MS analysis equipped with both collision-induced dissociation and electron transfer dissociation. From the electron transfer dissociation spectrum of the Lys-C digestion, we identified phospho-Ser-260, which resides in an R motif, RAS consensus sequence and is conserved among higher eukaryotes (Fig. 3B). Overexpression of wild-type RSK1 or RSK2, but not kinase inactive RSK1 (K94/447R) or RSK2 (K100/541R), enhanced CCTβ phosphorylation (Fig. 2B and supplemental Fig. S1A). In addition, S6K1 overexpression did not significantly increase CCTβ phosphorylation in the presence of activated RSK, although a small increase in phosphorylation in unstimulated cells was noted. Furthermore, reduction of RSK1 and RSK2 with RNAi suppressed CCTβ phosphorylation by PMA, indicating the requirement of RSK phosphotransferase activity for phosphor ester-induced CCTβ phosphorylation in intact cells (Fig. 2C).

**RSK Phosphorylates the CCTβ Subunit in Vivo and in Vitro**—To further demonstrate that CCTβ is phosphorylated in a RSK-dependent manner, we treated cells with several pharmacological inhibitors. EGF- or PMA-induced endogenous or ectopic CCTβ phosphorylation was sensitive to the MEK inhibitor UO126 and a recently developed RSK pan inhibitor BI-D1870 (31, 45) but resistant to the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 or the mammalian target of rapamycin (mTOR) inhibitor rapamycin, suggesting that CCTβ is phosphorylated by RSK, not Akt or S6k, in EGF- or PMA-stimulated HEK293E cells (Fig. 2A and supplemental Fig. S1B and C). Overexpression of wild-type RSK1 or RSK2, but not kinase inactive RSK1 (K94/447R) or RSK2 (K100/541R), enhanced CCTβ phosphorylation (Fig. 2B and supplemental Fig. S1A). In addition, S6K1 overexpression did not significantly increase CCTβ phosphorylation in the presence of activated RSK, although a small increase in phosphorylation in unstimulated cells was noted. Furthermore, reduction of RSK1 and RSK2 with RNAi suppressed CCTβ phosphorylation by PMA, indicating the requirement of RSK phosphotransferase activity for phosphor ester-induced CCTβ phosphorylation in intact cells (Fig. 2C).

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To verify that RSK phosphorylates CCT in vitro, RSK kinase assays were performed using the bacterial recombinant glutathione S-transferase (GST)-CCT fusion proteins as substrates. Activated HA-tagged RSK1 phosphorylated wild-type CCT protein, but did not phosphorylate the S260A protein, confirming that Ser-260 is the major site phosphorylated by RSK (Fig. 3C). These results indicate that RSK directly phosphorylates CCT Ser-260 in vitro and in intact cells.

S6K1 Participates in CCT Ser-260 Phosphorylation in the PI3K-mTOR Pathway—After confirming that RSK phosphorylates CCT on Ser-260 in PMA– or EGF-treated HEK293E cells, we examined whether CCT is phosphorylated by S6K
Phosphorylation of CCT by RSK and S6K

Upon insulin stimulation where RSK is only weakly activated (Fig. 4A). Because insulin is a potent activator for S6K, we predicted that S6K could participate in CCTβ Ser-260 phosphorylation when RSK activity was low.

To test this possibility, cells that were or were not pretreated with several pharmacological inhibitors were stimulated with insulin. Under these conditions, the MEK inhibitor U0126 was not effective at inhibiting insulin-induced CCTβ phosphorylation, while the mTOR inhibitor Rapamycin as well as PI3K inhibitor LY294002 suppressed the insulin-stimulated CCTβ phosphorylation (Fig. 4B). Reduction of S6K1 by siRNA inhibited CCTβ phosphorylation induced by insulin (Fig. 4C). Overexpression of S6K1 resulted in enhanced Ser-260 phosphorylation, but not for CCTβ S260A (Fig. 4D). In vitro kinase assays using HA-tagged S6K1 as an enzyme confirmed its ability to phosphorylate CCTβ (Fig. 4F). These results indicate that S6K1 is involved in CCTβ phosphorylation in insulin-treated HEK293E cells.

Although, insulin-stimulated CCTβ phosphorylation was substantially inhibited by rapamycin, conditions that do not inhibit Akt activation, we asked whether Akt could participate under conditions of overexpression. This analysis revealed that overexpression of Akt could also lead to increased CCTβ phosphorylation in HEK293E cells (Fig. 4D). The Akt-mediated CCTβ phosphorylation was not sensitive to rapamycin, although phosphorylation of ribosomal protein S6 was suppressed, indicating that S6K was inhibited (Fig. 4E). Furthermore, HA-tagged Akt can phosphorylate GST-CCTβ protein in vitro (Fig. 4F). Taken together, our findings suggest that the chaperonin CCTβ subunit can be targeted by S6K1 or Akt in the PI3K-mTOR pathway; however, Akt seems to play a minor role in HEK293E cells.

The Ras-MAPK and PI3K-mTOR Pathways Converge on CCTβ Phosphorylation in Different Cell Lines—To expand our observations beyond HEK293 cells, we examined CCTβ phosphorylation in several different cell lines. In all the cell lines examined, CCTβ phosphorylation was detected by pAS in response to PMA, EGF, and insulin. Responsiveness to anisomycin, a protein synthesis inhibitor and activator of stress kinases, was cell line-dependent (Fig. 5A).

To further examine the contribution of RSK, S6K, or Akt to Ser-260 phosphorylation, we characterized CCTβ phosphorylation in different cell lines. CCTβ Ser-260 was phosphorylated in the immortalized stable HMECs expressing FLAG-CCTβ (Fig. 5B). Although insulin alone induced CCTβ phosphoryla-
wild-type CCTβ were associated with the other CCT subunits such as CCTα and CCTγ in HMECs (Fig. 5B), and Ser-260 phosphorylation status did not alter subunit assembly in HMECs or in U2-OS (Fig. 5, C and D), indicating that Ser-260 is not required for CCTβ incorporation into the CCT complex. These results indicate that the Ras-MAPK and PI3K-mTOR pathways converge on the chaperonin CCT, and that the contribution of RSK and S6K to CCTβ Ser-260 phosphorylation is dependent on the cellular background.

Ser-260 Phosphorylation of CCTβ Is a Positive Regulator of Cell Proliferation—To address the physiological relevance of the RSK- or S6K-mediated phosphorylation of CCTβ, we determined whether Ser-260 phosphorylation affects CCT function in cells. Consistent with the findings by Grantham et al. (46), RNAi-mediated CCTβ knockdown was achieved in HMECs, which resulted in inhibition of cell proliferation (Fig. 6, A and B). Complementation of the shRNA-mediated CCTβ reduction with ectopically expressed shRNA-resistant murine CCTβ wild type or S260D recovered cell proliferation. Importantly, cells rescued with the S260A mutant CCTβ remained impaired in their rate of proliferation (Fig. 6, C and D). Thus Ser-260 phosphorylation of CCTβ is an important positive contributor to cell proliferation in HMECs.

DISCUSSION

The chaperonin CCT is part of a chaperone network linked to protein synthesis. The involvement of CCT in cytoskeletal organization by folding actin and tubulin or cell cycle progression by promoting the folding of several cell cycle regulators has been proposed (46), but how CCT contributes to protein folding for newly synthesized proteins in cells remains controversial. Some results indicate that ~5–10% of newly synthesized proteins flow through CCT (29, 30), whereas other studies have shown that CCT interacts with only a small amount (1%) of total proteins (23, 46).

Through the use of the phospho-Akt substrate antibody, protein purification, and mass spectrometry, we have identified the CCT β subunit as a novel physiological target of growth factor-
insulin-, and nutrient-regulated signals. Specifically, we show that CCTβ is targeted for phosphorylation by the Ras and PI3K/mTOR pathways. We also show that phosphorylation of the CCTβ subunit is an important contributor to the regulation of cell proliferation in mammalian cells. The ERK-regulated protein kinase RSK is the dominant kinase that phosphorylates CCTβ when HEK293E cells are treated with EGF or the tumor promoter PMA (Figs. 2 and 4A). Upon insulin stimulation of HEK293E cells, however, CCTβ phosphorylation was dependent on PI3K-mTOR activation (Fig. 4B) as RSK is weakly activated. Under these conditions S6K, but not Akt, regulates CCTβ phosphorylation in insulin-treated HEK293E cells.

We demonstrate that growth factor-mediated CCTβ phosphorylation occurs at Ser-260, a conserved amino acid lying within the consensus Akt motif RXRXxpS. Mass spectrometry and mutagenesis analysis revealed that RSK and S6K1 phosphorylate CCTβ Ser-260 in vitro and in intact cells (Figs. 3 and 4). Consistent with the findings in HEK293E cells, CCTβ phosphorylation occurs in various cell lines such as HMECs, osteosarcoma U2-OS cells, breast normal epithelial MCF10A cells, and human breast adenocarcinoma MDA-MB-231 cells (Fig. 5 and data not shown). The contribution of RSK or S6K is dependent on the cellular background and agonist used. Although Akt weakly phosphorylated CCTβ in vitro, overexpression of Akt led to a measurable induction of CCTβ Ser-260 phosphorylation in HEK293 cells, and its phosphorylation was not sensitive to S6K inhibition (Fig. 4, D and E). A significant contribution of Akt to CCTβ Ser-260 phosphorylation, however, was not observed under physiological conditions in several cell lines tested (Figs. 4B, 5C, and 5E). Thus, we conclude that RSK and S6K are largely responsible for coordinately regulating CCTβ phosphorylation in mammalian cells.

Stimulation of CCTβ phosphorylation in serum-starved HEK293 cells treated with PMA occurred within 3 min after stimulation, peaked at 15 min to 2 h, and lasted for over 6 h or well into the G1 phase of the cell cycle (supplemental Fig. S1D). In addition to the mitogen- and phorbol ester-stimulated, post-translational modification of CCTβ that we describe here, two of the eight subunits, CCTβ and CCTγ, have been identified as mitosis-specific phosphorylated proteins. The exact phosphorylation sites and their functional relevance, however, have not been determined (47). Thus, the chaperonin CCT may be subjected to multiple signals throughout the cell cycle, and these modifications may also play an important role in regulating CCT function.

Previous studies have suggested that CCTβ expression is required for normal cell proliferation (46). CCTβ has also been reported to be overexpressed in colorectal adenocarcinomas, and its overexpression is associated with poor prognosis (48). Although unable to determine if CCTβ phosphorylation affects its specific or general protein folding function, we have demonstrated that RSK- and S6K-dependent phosphorylation of the chaperonin CCTβ correlates with enhanced cell proliferation rates. As published, CCTβ depletion did reduce the rate of cell proliferation, and we show that this can be rescued with an RNAi-insensitive wt-CCTβ. However, although CCTβ S260A normally associated with the other CCT subunits (Fig. 5, B and C), HMECs expressing S260A failed to complement the impaired phenotype (Fig. 6, C and D), indicating that Ser-260 phosphorylation is an important contributor to cell division. Further analysis is required to reveal at a molecular level, how CCTβ phosphorylation directly contributes to cell growth and proliferation.

Structural information may provide some clues. The chaperonin CCT exists as multimers (800–1000 kDa) composed of eight different subunits arranged in two back-to-back rings (49) (Fig. 7A). Each CCT subunit is composed of three different domains: the apical domain with the peptide binding motif; the equatorial domain, important for intra- and inter-ring interac-

**FIGURE 6.** CCTβ Ser-260 phosphorylation is required for optimal cell proliferation. A, HMECs with stable shRNA-mediated CCTβ knockdown were lysed, and immunoblot analysis was performed to determine the level of CCTβ knockdown. Two distinct shRNA constructs were used. B, HMECs with stable shRNA-mediated CCTβ knockdown and vector-control cells were plated, and the cell number was determined at the indicated times after cell plating. Each value represents the mean ± S.D. of triplicate determinations from a representative experiment. C, stable shRNA1-CCTβ knockdown cells, shRNA1-CCTβ knockdown cells complemented with murine CCTβ WT, S260A, or S260D were lysed, and immunoblot analysis was performed. D, stable shRNA1-CCTβ knockdown cells complemented with murine CCTβ WT, S260A, or S260D were plated, and the cell number was determined at the indicated times after plating. Each value represents the mean ± S.D. of triplicate determinations from a representative experiment. Similar results were obtained in at least three independent experiments.
Prefoldin associates with newly synthesized actin and tubulin, and cryoelectron microscopy has examined the interaction between CCT and prefoldin in vitro (59). Hsp70 and CCT contribute to the folding of a range of proteins, including Von Hippel-Lindau and the polyglutamine repeats in huntingtin (28, 55, 61). Hsp70 was shown to form a stable complex with CCT in vitro and Hsp70-CCT complex showed higher protein folding activity. Furthermore, the docking site is predicted to be in the CCT β apical domain (62). This suggests the possibility that CCT β Ser-260 phosphorylation may be important for regulation of its interaction with specific proteins in intact cells.

Upon growth factor stimulation a variety of AGC kinases are activated downstream of Ras and PI3K-mTOR, including RSK, S6K, and/or Akt. These kinases help to coordinate gene transcription and successive protein translation by phosphorylating transcription factors, immediate-early gene products, and various translational regulators, including the tumor suppressors TSC2 and PDCD4, raptor, eukaryotic initiation factor 4B, and the 40S ribosomal protein S6. Improper regulation of protein synthesis has been linked to a variety of metabolic diseases and cancer. We now demonstrate that the eukaryotic chaperonin CCT is targeted by Ras-MAPK and PI3K-mTOR pathways and that RSK and S6K relay a mitogenic signal to the eukaryotic chaperonin CCT β. Future studies will be aimed at determining the exact role of CCT and its individual subunits during physiological or pathophysiological conditions and its regulation by AGC kinases.

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