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Calcium-Dependent Interaction of Calmodulin with Human 80S Ribosomes and Polyribosomes

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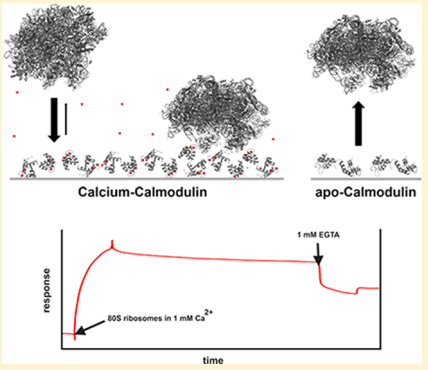
Supporting Information

ABSTRACT: Ribosomes are the protein factories of every living cell. The process of protein translation is highly complex and tightly regulated by a large number of diverse RNAs and proteins. Earlier studies indicate that Ca^{2+} plays a role in protein translation. Calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein, regulates a large number of proteins participating in many signaling pathways. Several 40S and 60S ribosomal proteins have been identified to interact with CaM, and here, we report that CaM binds with high affinity to 80S ribosomes and polyribosomes in a Ca^{2+}-dependent manner. No binding is observed in buffer with 6 mM Mg^{2+} and 1 mM EGTA that chelates Ca^{2+}, suggesting high specificity of the CaM−ribosome interaction dependent on the Ca^{2+} induced conformational change of CaM. The interactions between CaM and ribosomes are inhibited by synthetic peptides comprising putative CaM-binding sites in ribosomal proteins S2 and L14. Using a cell-free *in vitro* translation system, we further found that these synthetic peptides are potent inhibitors of protein synthesis. Our results identify an involvement of CaM in the translational activity of ribosomes.



Protein synthesis is one of the most complex and tightly regulated cellular processes in all organisms. Regulation involves several steps, including a multitude of proteins and RNAs, and is tightly integrated with a variety of cellular pathways. The process of translation is carried out by ribosomes and their associated factors. In eukaryotic cells, protein translation takes place at the 80S ribosome, which consists of a small 40S subunit [containing 18S rRNA and ∼33 different proteins] and a large 60S subunit [containing 5S rRNA, 5,8 rRNA, 28S rRNA, and ∼49 different proteins].[1−3] Translation is initiated at the start codon of mRNA by the assembly of the 40S ribosomal subunit with an initiator tRNA and the 60S subunit to form a translationally active 80S ribosome.[4−7] After initiation, repetitive codon-directed addition of aminoacyl-tRNAs leads to elongation of the polypeptide chain until the final mRNA stop codon is reached and the polypeptide dissociates from the ribosome.[8−12] In recent years, several studies have provided strong evidence that Ca^{2+} plays a crucial role in the regulation of eukaryotic protein translation.[13−18] Kumar et al.,[19] found that calmodulin antagonists block the initiation of protein synthesis, indicating that CaM or a very similar Ca^{2+}-binding protein might be involved in the process. CaM is a ubiquitously expressed Ca^{2+}-binding protein that is involved in a large number of regulatory processes and is highly conserved throughout eukaryotic organisms.[20−22] Calmodulin is a major transmitter of Ca^{2+} signals in cells and interacts with a multitude of different proteins in response to intracellular [Ca^{2+}] changes and thereby participates in various signaling pathways that regulate processes such as cell proliferation, learning and memory, growth, and movement.[23−29] A number of free 40S and 60S ribosomal proteins and other proteins associated with ribosomes have been found to interact with CaM.[30−41] These findings support the idea that CaM may play a role in protein synthesis.[19] However, the interaction of CaM with functional 80S ribosomes has to our knowledge not been demonstrated. In the present study, we analyzed the interaction of CaM with purified 80S ribosomes and polyribosomes using surface plasmon resonance (SPR) technology. Our data imply that calmodulin interacts, in a Ca^{2+}-dependent manner, with intact 80S ribosomes and polyribosomes via ribosomal proteins or other ribosome-associated proteins. Further, we identified putative CaM-binding sites in two previously identified ribosomal CaM targets, rpS2 and rpL14, and synthesized these as linear peptides. SPR, isothermal titration calorimetry (ITC), and inhibition of translation activity studies show Ca^{2+}-dependent binding of

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these peptides to CaM, inhibition of CaM-binding to 80S ribosomes, and inhibition of protein translation.

EXPERIMENTAL PROCEDURES

HeLa-Cell Culture. HeLa S3 cells were purchased from the Mammalian Protein Expression (MPE) Core Facility at the University of Gothenburg.

Preparation of Cytoplasmic HeLa-Cell Lysates. For cell lysis, $2.17-3.07 \times 10^9$ HeLa-cells were incubated for 10 min on ice in lysis buffer (50 mM Tris, 250 mM sucrose, 250 mM KCl, 6 mM $MgCl_2$, protease inhibitor cocktail (Roche), 2 mM DTT, 5 U/mL DNase, and 5 U/mL RNase inhibitor at pH 7.5). The lysed cells were then gently homogenized and centrifuged for 10 min at 750*g* and 4 °C to remove the mitochondria. The postmitochondrial supernatant was centrifuged for 10 min at 12,500*g* at 4 °C to remove the nuclei. The obtained cytoplasmic lysate was directly used for further purification of polyribosomes or 80S ribosomes.

Purification of Polyribosomes and 80S Ribosomes. Polyribosomes and 80S ribosomes were purified from the cytoplasmic HeLa-cell lysate via sucrose gradient centrifugation. For 80S ribosome purification, the KCl concentration of the lysate was adjusted to 0.5 M, and all sucrose centrifugation steps were performed in the presence of 0.5 M KCl to disrupt most interactions between ribosomes and other proteins.[42] For polyribosome purification, the KCl concentration was 150 mM. The lysates were layered on a 1 M sucrose cushion (1 M sucrose in buffer A (50 mM Tris, 150 or 500 mM KCl, 6 mM $MgCl_2$, protease inhibitor cocktail (Roche), 1 mM DTT, 5 U/mL DNase, and 5 U/mL RNase inhibitor at pH 7.5) and centrifuged for 4 h at 12,500*g* at 4 °C. The obtained pellet was washed, resuspended in buffer A, and centrifuged through a linear (10−30%) sucrose gradient (prepared in buffer A) for 10 h at 100,000*g* and 4 °C. The sucrose gradient was fractionated from the bottom of the centrifuge tubes, and each fraction was analyzed by SDS−PAGE and absorbance at 280 and 254 nm (Figure S1, Supporting Information). The polyribosomal and 80S ribosomal fractions were pooled and dialyzed against 10 mM Tris at pH 7.5, 150 mM KCl, and 6 mM $MgCl_2$. The concentration of the purified 80S ribosomes was determined by absorbance measurements at 260 nm. Polyribosomes and 80S ribosomes were directly used for the experiments or snap-frozen in liquid nitrogen and stored at −80 °C until further use.

Expression and Purification of Recombinant Human CaM and Mutant S17C-CaM. Human wild type CaM was expressed from the PetSac-vector, a modified Pet3a-vector containing a synthetic gene with codons optimized for expression in *E. coli*, and purified as previously described.[43] Using primers containing the desired base change, the mutant S17C-sequence variant was amplified from the PetSac-vector containing the synthetic CaM gene, introduced into the PetSac-vector and expressed and purified as described.[33,43] Apo-CaM was obtained by chelating Ca^{2+} with EDTA. CaM was subsequently separated from EDTA by using a G25 gel filtration column (Amersham Bioscience). The purity of CaM was confirmed by agarose− and SDS−polyacrylamide gel electrophoresis under Ca^{2+} and Ca^{2+}-free (EDTA) conditions and by ^1H NMR spectroscopy. The decalcification was analyzed with Ca^{2+} titrations in the presence of Quin2[44] (residual Ca^{2+} less than 0.04 molar equiv, which is less than 1% of full saturation).

CaM-Sepharose Pull down Assays. Activated thiol-sepharose 4B (GE Healthcare) was swollen according to the manufacturer's instructions and washed with CaM-coupling buffer (0.1 M Tris at pH 7.5, 0.5 M NaCl, and 1 mM EDTA). S17C-CaM was incubated with the swollen gel and stirred for 2−4 h. Nonbound S17C-CaM was removed by washing with CaM-coupling buffer, and nonreacted thiol groups were blocked by washing with 1 mM 2-mercaptoethanol in 50 mM sodium acetate at pH 4.5. Subsequently, the gel material was equilibrated with buffer B (10 mM Tris at pH 7.5, 150 mM KCl, 6 mM $MgCl_2$, and 1 mM $CaCl_2$). To provide control resins, an equal amount of activated thiol sepharose was prepared with the EF-hand protein calbindin D_{9k} with a GGC extension[45] or without protein. Five hundred microliters of purified ribosomes (A_{260} = 4) or polyribosomes were complemented with 1 mM $CaCl_2$ and incubated for 1 h with equal amounts of either control-Sepharose or CaM-Sepharose. After incubation, nonbound polyribosomes and ribosomes were removed by washing with buffer B. Bound polyribosomes and ribosomes were eluted with EGTA-buffer (10 mM Tris at pH 7.5, 150 mM KCl, 6 mM $MgCl_2$, and 1 mM EGTA). Afterward, the EGTA-eluted proteins were precipitated by trichloroacetic acid and analyzed by SDS−PAGE and mass spectrometry.

Mass Spectrometry. Proteins were separated by SDS−PAGE and Coomassie-stained. Stained bands were excised and used for in-gel digestion of proteins as described.[46] Mass spectra for the proteolytic digests were acquired using a 4700 proteomics analyzer (Applied Biosystems, Framingham, MA) mass spectrometer in positive reflector mode. For MS and tandem MS (MS-MS) analyses, approximately 1,000 and 3,000 single laser shot spectra were summed up, respectively. Interpretation of mass spectra was performed using Mascot (www.matrixscience.com).

Identification of Putative CaM-Binding Sites. To identify putative CaM-binding sites in rpS2, rpSL9, and rpSL14, the corresponding protein sequences were analyzed using an algorithm developed by Ikura et al. (http://calcium.uhnres.utoronto.ca) along with a manual inspection of the sequences.

Sequence Alignments. The sequences of the human and orthologous ribosomal proteins (rp) S2, L9, and L14 were obtained from UniProt (http://www.uniprot.org), the Protein (http://www.rcsb.org/pdb), and the NCBI Data Bank (http://www.ncbi.nlm.nih.gov/RefSeq), and global multiple sequence alignments were performed using the ClustalW algorithm (http://www.ebi.ac.uk).

Visualization of Ribosomal Proteins in the 80S Ribosome Structure. To visualize ribosomal proteins and the identified putative CaM-binding sites in a 3D model structure of the 80S ribosome, we used the available X-ray structure of the yeast 80S ribosome (PDB ID: 3O5H and 3O2Z)[47] and the software Pymol.

Synthesis and Purification of Putative CaM-Binding Sites. The peptides corresponding to the identified putative CaM-binding sites from human rpS2, rpL9, and rpL14 were synthesized using a standard Fmoc-based solid-phase protocol with an Applied Biosystems 433A or a PS3 Protein Technologies Peptide Synthesizer as described.[33] The crude peptides were then purified by reversed phase HPLC as previously described.[33] All peptides were synthesized with capped ends, with an acetylated N-terminus and through employing the PAL resin, a C-terminal carboxamide. The identity of each peptide was verified by ESI MS performed by Texas A&M University, College Station, TX.

Isothermal Titration Calorimetry. The interactions of the putative CaM binding peptides with wild-type CaM were analyzed by isothermal titration calorimetry (ITC) using a VP-ITC microcalorimeter (MicroCal, Piscataway, NJ). The data were analyzed with the Origin7 software from MicroCal using a 1:1 binding model. Wild-type CaM and peptides were prepared in 10 mM Tris and 150 mM KCl at pH 7.5 with either 1 mM $CaCl_2$ or 1 mM EDTA and degassed prior to each measurement. The CaM concentration was determined by absorbance using ε = 3,200 L mol^{-1} cm^{-1} and the concentrations of peptide stocks by amino acid analysis after acid hydrolysis (analysis purchased from BMC, Uppsala, Sweden). Each peptide was titrated from a 200 μM stock solution into 10 μM wild-type CaM at 25 °C with an initial injection of 5 μL followed by 29 injections of 10 μL of peptide solution. The equilibration time between each injection was 5 min.

Surface Plasmon Resonance Spectroscopy with Immobilized CaM. All SPR studies were performed using a Biacore 3000 instrument. S17C-CaM was immobilized to a CM5 chip using ligand thiol disulfide exchange coupling as described.[33] For control experiments, calbindin D_{9k}[44] was immobilized using the same method. Blank channels for negative control were prepared by omitting S17C-CaM in the coupling step. To analyze interactions of ribosomes with immobilized CaM, 50 μL of 80S ribosomes (1 μM) or polyribosomes in 10 mM Tris, 150 mM KCl, 6 mM $MgCl_2$, 0.005% (v/v) Tween 20, 1 mM $CaCl_2$, or 1 mM EGTA at pH 7.5 was injected. The same buffer was used as running buffer. The flow rate was 5 μL/min throughout the experiment. The interaction of rRNA with immobilized CaM was performed under the same conditions using RNA purified by ethanol precipitation from ribosomal or polyribosomal preparations and the concentration adjusted to give the same absorbance at 254 nm as the original preparations. Peptide competition experiments were performed under the same conditions with the difference that ribosomes were mixed with 100-fold molar excess of peptide before injecting.

***In Vitro* Translation Assays.** *In vitro* translation assays were performed in the absence or presence of different peptides or trifluoperazine (TFP) concentrations using a 1-Step Human *in Vitro* Protein Expression Kit (Pierce) as described by the manufacturer with an mRNA for green fluorescent protein (GFP-mRNA) as a template. Measurements were performed in a 96-well plate, and the expression of GFP was monitored at 37 °C by recording the fluorescence as a function of time using a Fluostar Optima or Galaxy plate reader (BMG Labtech, Offenburg, Germany) with a 482 nm excitation filter and a 512 nm emission filter. The data is expressed as the percentage inhibition of GFP fluorescence compared to samples without peptide at the end of the experiment.

RESULTS

Ca^{2+}-Dependent Interactions of Ribosomes with CaM-Sepharose. To analyze the interaction of CaM with 80S ribosomes, we performed CaM pull down assays by mixing CaM-sepharose with freshly purified 80S ribosomes in the presence of Ca^{2+}. Proteins that bound to Ca^{2+}-CaM-sepharose were eluted with EGTA and separated by SDS−PAGE (Figure 1A). The eluted proteins display a pattern of gel bands highly similar to that of intact 80S ribosomes (see Figure S1, Supporting Information), indicating that the ribosomal complexes bind to CaM-sepharose in a Ca^{2+}-dependent manner. In contrast, no binding was observed for the control resin. Furthermore, no binding was observed for calbindin D_{9K}-sepharose showing that ribosomes bind specifically to CaM. To identify the specific ribosomal proteins that bind to CaM-sepharose, we analyzed selected protein bands by mass spectrometry. Using this method, we identified several 60S ribosomal proteins as well as three 40S ribosomal proteins in the 80S ribosomal fraction (Figure 1 and Table 1). These results indicate that intact 80S ribosomes bind to Ca^{2+}-CaM. The same experiment was carried out with purified polyribosomes, (i.e., complexes of several ribosomes on the same mRNA (Figure 1B)), and we observed Ca^{2+}-dependent binding of polyribosomal proteins to CaM-sepharose with the eluate displaying a protein pattern similar to that of intact polyribosomes. We could further identify 60S and 40S ribosomal proteins as well as other proteins associated with ribosomes and polyribosomal complexes via mass spectrometry. This suggests that ribosomes interact with CaM-sepharose also when present in polyribosomal complexes (Figure 1 and Table 1).

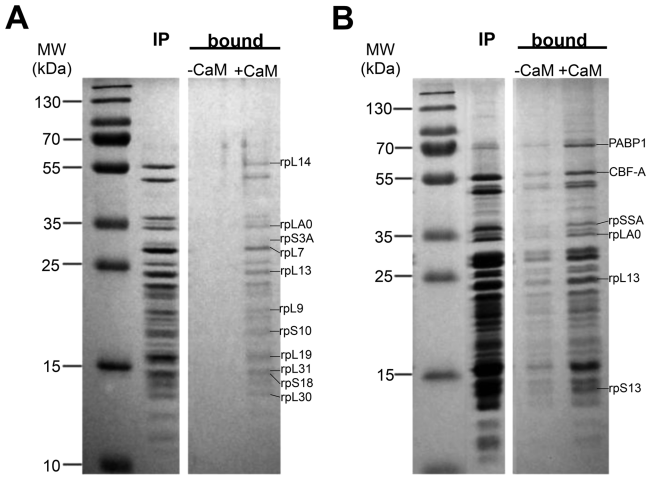

Figure 1. Ca^{2+}-dependent CaM-pull down assays. Activated thiol-sepharose was immobilized with S17C-CaM, incubated with purified 80S ribosomes or polyribosomes in the presence of 1 mM Ca^{2+} and 6 mM $MgCl_2$,and eluted with 1 mM EGTA. (A) CaM-pull down assays with 80S ribosomes. (B) CaM pull down assays with polyribosomes. IP, purified 80 ribosomes or polyribosomes; bound, proteins that bound Ca^{2+}-dependently to the resin. −CaM: control without coupled S17C-CaM. +CaM: with S17C-CaM coupled to sepharose. Proteins that were identified by mass spectrometry are labeled and listed in Table 1.

SPR Studies of CaM Interaction with Ribosomes. The results of the SPR experiments are shown in Figure 2. Human S17C-CaM was coupled via the cysteine thiol to a sensor chip, and the binding and dissociation of the purified human ribosomes was studied. The resulting sensorgram indicates that ribosomes bind with high affinity to immobilized CaM in the presence of Ca^{2+} (K_D = 37 nM; $K_A = 2.7 \times 10^7$ M^{-1}), whereas no binding was observed in the absence of Ca^{2+} (Figure 2A). In both cases, 6 mM Mg^{2+} was present. The results show that Mg^{2+}-binding to CaM is not sufficient to yield high affinity for ribosomes, suggesting a reliance on the Ca^{2+}-induced conformational change of CaM. In control experiments with immobilized calbindin D_{9k}, no binding was detected under Ca^{2+} or Ca^{2+}-free conditions (Figure 2C), indicating that ribosome binding does not occur merely due to a negatively charged EF-hand protein being immobilized in the dextran matrix. A

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Table 1. Ribosomal and Polyribosomal Proteins Identified by Mass Spectrometry and MASCOT Search[a]

80S ribosomes		
label	protein	function
rpL14	60S ribosomal protein L14	RNA and protein binding; structural constituent of ribosome
rpLA0	60S acidic ribosomal protein P0	
rpL7	60S ribosomal protein L7	
rpL13	60S ribosomal protein L13	
rpL9	60S ribosomal protein L9	
rpL19	60S ribosomal protein L19	
rpL31	60S ribosomal protein L31	
rpL30	60S ribosomal protein L30	
rpS3A	40S ribosomal protein S3A	
rpS10	40S ribosomal protein S10	
rpS18	40S ribosomal protein S18	
Polyribosomes		
label	protein	function
PABP1	polyadenylate-binding protein 1	binds the poly(A) tail of mRNA
CBF-A	CCAAT-binding factor subunit A	processing of mRNA
rpLA0	60S ribosomal protein LA0	RNA and protein binding; structural constituent of ribosome
rpL13	60S ribosomal protein L13	
rpS13	40S ribosomal protein S13	

[a]Proteins that bound Ca^{2+}-dependently to CaM-sepharose were separated by SDS–PAGE (Figure 1), and prominent protein bands were excised and analyzed by mass spectroscopy. Listed are the names and functions of proteins that are labeled in Figure 1.

second experiment revealed that the binding of ribosomes in the presence of Ca^{2+} could be reversed by chelating Ca^{2+} with an injection of 1 mM EGTA (Figure 2B). This observation further proves the Ca^{2+} dependency of CaM interaction with ribosomes. With immobilized CaM, we also recorded the high affinity binding of polyribosomes in the presence of Ca^{2+} and no binding in the absence of Ca^{2+} (Figure 2D). Thus, ribosomes seem to bind to Ca^{2+}-CaM also when present in a polyribosomal complex. The results of the SPR studies confirm the observations made using pull down assays. When ribosomal and polyribosomal RNA were injected over the immobilized CaM, a much weaker response was observed compared to that in ribosomes or polyribosomes with the same RNA concentration (Figure S2, Supporting Information) showing that CaM interacts directly with ribosomal proteins or proteins associated with ribosomes rather than the RNA in these complexes.

Putative CaM-Binding Sites. Ribosomal proteins S2, L9, and L14 are among the previously identified CaM targets.[32,33] Using a web-based search engine[48] combined with multiple sequence alignment and manual inspection, we identified putative CaM-binding sites in the human protein sequences of these three proteins as follows: residues 114–131 of rpS2, KQTRAGQRTRFKAFVAIG; residues 161–178 of rpL9, IQQATTVKNKDIRKFLDG; and residues 67–94 of rpL14, SAHQKYVRQAWQKADINTKWAATRWAKK. These sequences are highly conserved among different species (Figure 3A). In the high-resolution structure of yeast 80S ribosome,[47] the proteins corresponding to human rpS2, rpL9, and rpL14 are found at the surface of the ribosome (Figure 3B). The ribosomal protein S2 is located on the solvent accessible surface of the 40S subunit[49,50] with the putative CaM-binding site in an extended loop that penetrates inside a pocket formed of 40S rRNA. The ribosomal protein L9 consists of two domains and is located at the surface of the 60S subunit. Here, the putative CaM-binding site is located in the C-terminal domain and is part of a loop directed to the rRNA of the 60S subunit. The ribosomal protein L14 is located at the surface of the 60S subunit and exposed to the solvent. It has a C-terminal loop region that contains two small α-helices. The putative CaM-binding site of rpL14 is present in the second small C-terminal helix and extends to loops in the N- and C-terminal direction. The location of the ribosomal CaM-binding proteins and their putative CaM binding sites at the surface of the ribosome may facilitate CaM binding to these proteins when present in the functional ribosome.

Interaction of Synthetic Peptides with CaM. To investigate the binding properties of the putative CaM-binding sites of the ribosomal proteins, we synthesized linear peptides of the corresponding sequences and tested their Ca^{2+}-dependent interactions with CaM using isothermal titration calorimetry (ITC; Figure 4). The peptides were titrated into wild-type CaM in the presence or absence of Ca^{2+}. Titrations with the rpS2 peptide revealed an exothermic process both under Ca^{2+} and Ca^{2+}-free conditions; however, the generated heat is smaller in the absence of Ca^{2+} (Figure 4A and B). This indicates that CaM interacts with the rpS2 peptide in a Ca^{2+}-dependent manner. Fitting a 1:1 stoichiometric binding model to the data, yields a K_D value of 0.5 μM for the rpS2-peptide binding to Ca^{2+}-CaM. With the rpL14 peptide, we could observe an exothermic process when Ca^{2+} was present and no change in enthalpy when Ca^{2+} was chelated by EDTA (Figure 4E and 4F). Fitting to the data revealed that this peptide binds to Ca^{2+}-CaM with a K_D of 1.2 μM, thus with slightly lower affinity than the rpS2 peptide. Titrations with the rpL9 peptide revealed no heat either in the presence or the absence of Ca^{2+} (Figure 4C and 4D). The signals obtained with this peptide were of the same magnitude as observed when the peptide was injected into buffer. This suggests that the peptide has no CaM-binding activity or alternatively that ΔH may be zero.

Peptide Competition with Intact Ribosomes. To further analyze the specificity of the CaM–ribosome interaction and to confirm the interaction of CaM with the putative CaM-binding sites, we carried out competition binding studies with the synthetic peptides and ribosomes using SPR. S17C-CaM was coupled via the cysteine thiol group to a sensor chip, and the binding of the purified human ribosomes was studied in the presence or absence of peptides under Ca^{2+}-conditions (Figure 5). The resulting sensorgrams show that in the presence of a 100-fold molar excess of rpS2 peptide, the binding of ribosomes to CaM is completely eliminated. This shows that the identified CaM-binding site in rpS2 competes with 80S ribosomes for binding to CaM. The same effect was observed with the rpL14 peptide. These findings support the ITC results for both peptides and confirm their binding to CaM. To assess the specific nature of this interaction, we used a 100-fold molar

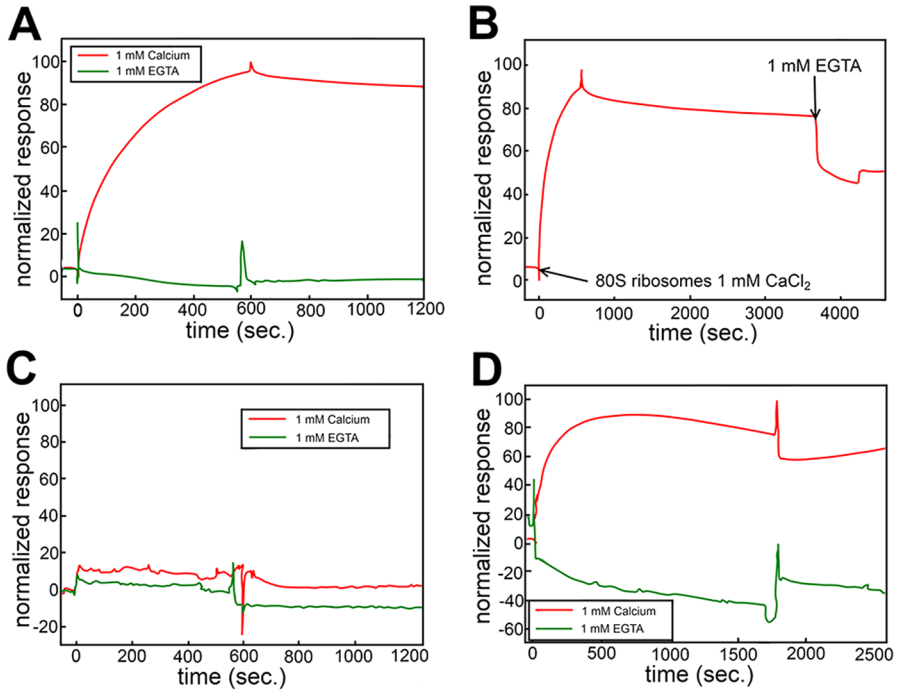

Figure 2. SPR validation of CaM interactions with 80S ribosomes and polyribosomes. Sensorgrams were recorded in the presence of 1 mM $CalCl_2$ (red) or 1 mM EGTA (green). All samples contain 6 mM $MgCl_2$. (A) Interactions of 80S ribosomes with immobilized S17C-CaM. (B) Binding of 80S ribosomes to immobilized S17C-CaM in the presence of 1 mM Ca^{2+} followed by an injection of 1 mM EGTA. (C) Ribosomes injected over immobilized calbindin D_{9k}. (D) Interactions of polyribosomes with immobilized S17C-CaM.

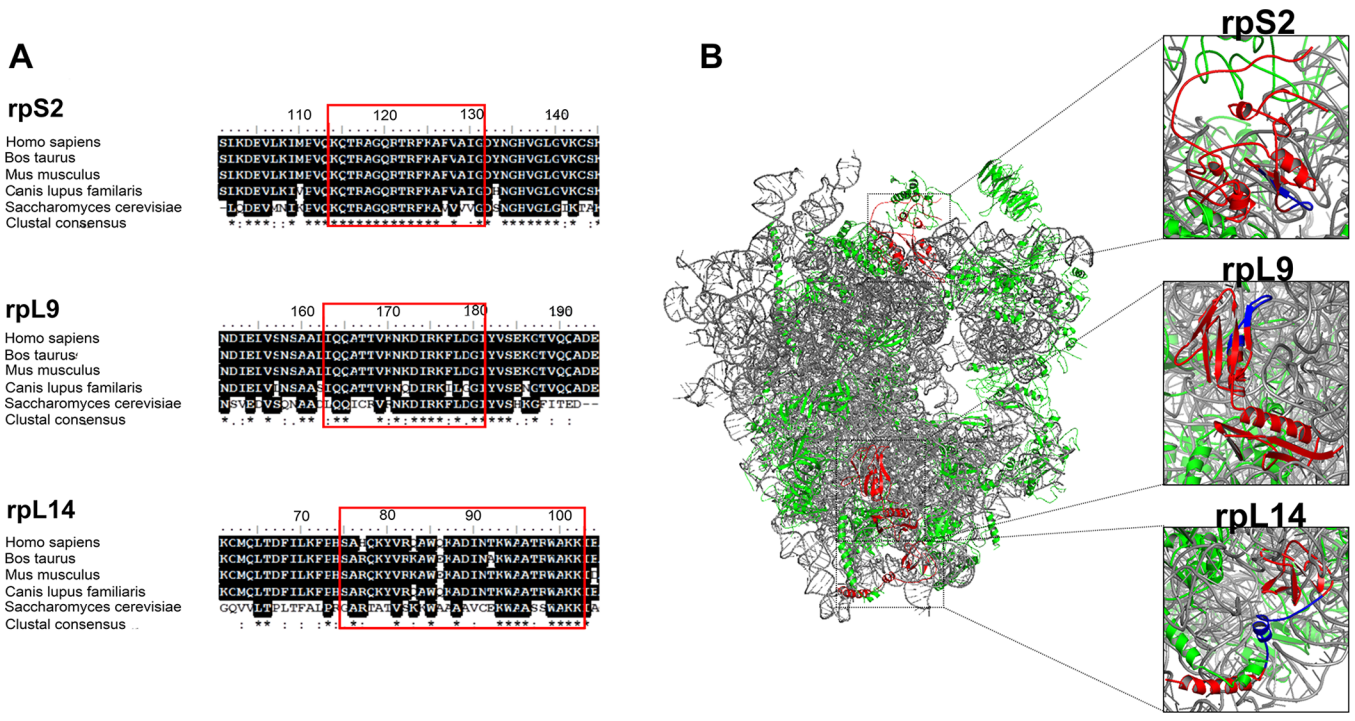

Figure 3. Putative CaM-binding sites in the ribosomal proteins S2, L9, and L14. (A) Multiple amino acid sequence alignment of human rpS2, rpL9, and rpL14 with orthologous proteins (rpS2, P15880.2, AAI02228.1, AAH91730.1, 2ZKQ_e, and 3O2Z_B; rpL9, P32969, AAW82089.1, AAH13165.1, XP_851404.1, and CAA42746.1; rpL14, P50914.4, Q3T0U2, Q569Z0, XP_534230.1, and 3O5H). Putative CaM-binding sequences are marked with red boxes. (B) 80S ribosome structure (PDB ID: 3O58/3O2Z). Ribosomal RNA is presented in gray, ribosomal proteins are presented in green. Ribosomal proteins S2, L9, and L14 are highlighted in red. rpS2: detailed view of ribosomal protein S2 when located in the 40S subunit of the 80Sribosome. rpL9: detailed view of ribosomal protein L9 when located in the 60S subunit of the 80S ribosome. rpL14: detailed view of ribosomal protein L14 when located in the 60S subunit of the 80Sribosome. The identified putative CaM-binding sites of rpS2, rpL9, and rpL14 are colored in blue.

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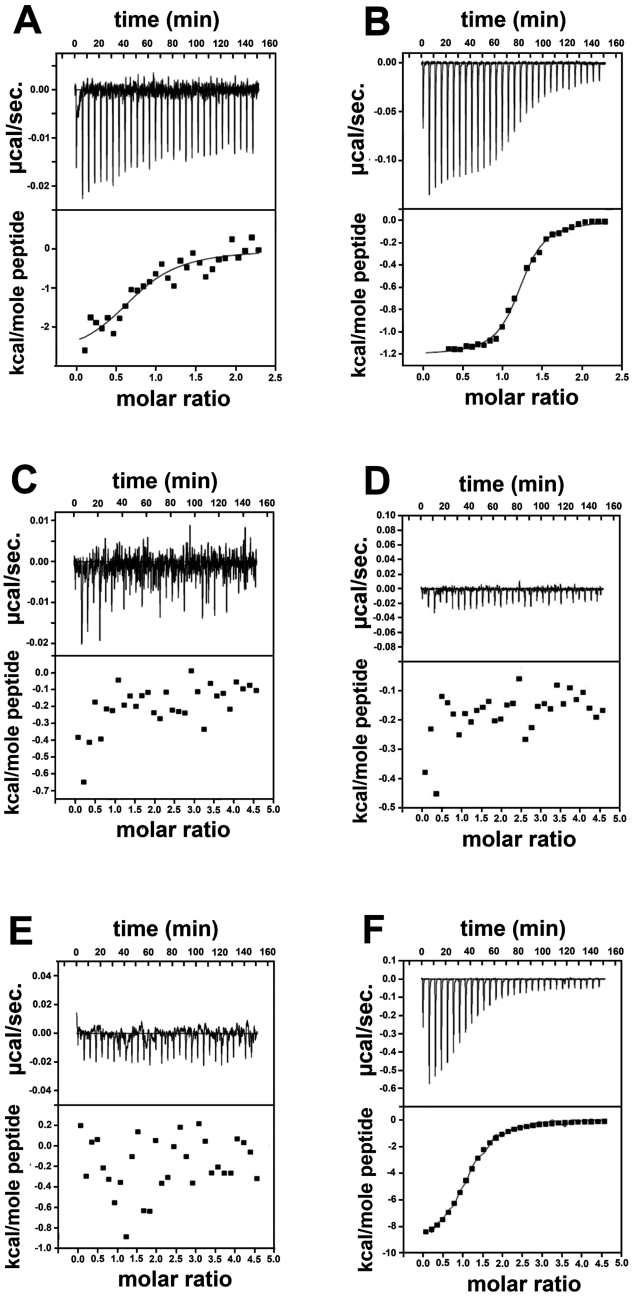

Figure 4. Isothermal titration calorimetry of ribosomal peptide binding to wild type CaM. Peptides were titrated from a 200 μM stock solution into 10 μM CaM at 25 °C. (A) Titrations with rpS2-peptide in the presence of 1 mM EDTA. (B) Titrations with rpS2-peptide in the presence of 1 mM Ca^{2+}. (C) Titrations with rpL9-peptide in the presence of 1 mM EDTA. (D) Titrations with rpL9-peptide in the presence of 1 mM Ca^{2+}. (E) Titrations with rpL14-peptide in the presence of 1 mM EDTA. (F) Titrations with rpL14-peptide in the presence of 1 mM Ca^{2+}.

excess of the CaM-binding peptide from the smooth muscle myosin light chain kinase (smMLCK), a protein that is known to bind to Ca^{2+}-CaM with two bulky (W5 and L18) residues serving as anchors.[51] We also observed competition, but in contrast to rpS2 and rpL14, this peptide did not lead to a total loss of 80S ribosome binding, and there was still some detectable binding signal via SPR (Figure 5). This suggests that smMLCK interacts with a different binding site of CaM and/or leads to different conformational changes in CaM upon binding

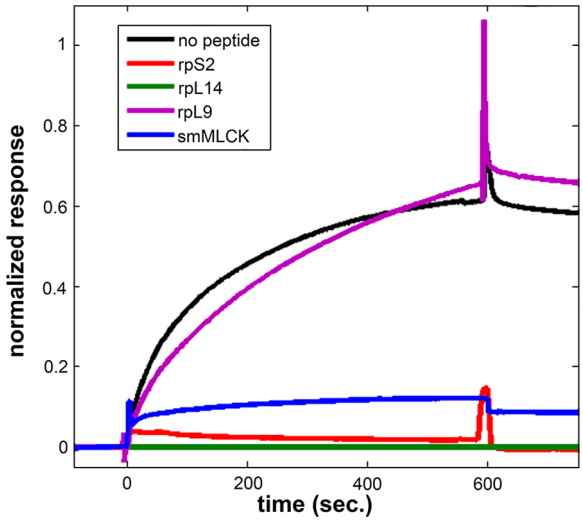

Figure 5. 80S ribosome binding to CaM in the presence of synthetic ribosomal CaM binding peptides. Interactions of 80S ribosomes to immobilized S17C-CaM were recorded in the presence of 100-fold molar excess of rpS2 peptide (red line), rpL9 peptide (magenta line), rpL14 peptide (green line), smMLCK peptide (blue line), and without peptide (black line). All measurements were performed in the presence of 1 mM Ca^{2+} and 6 mM Mg^{2+}.

compared to rpS2 or rpL14. With the rpL9 peptide, we observed no competition. Together with the lack of signal in ITC, this peptide does not seem to represent the CaM-binding site of rpL9.

Inhibition of Protein Synthesis by CaM-Binding Peptides. The relative abilities of the ribosomal CaM-binding peptides rpS2 and rpL14 and the smMLCK-peptide to inhibit protein translation were analyzed using a human cell-free *in vitro* translation assay using an mRNA for green fluorescent protein (GFP-mRNA) as a template. The translational activity at different peptide concentrations was measured through GFP-fluorescence intensity (Figure 6A–C). Cell-free translation was found to be inhibited by all three peptides at concentrations between 5 and 500 μM leading to a decrease in translational activity. To verify that the inhibition of protein synthesis by rpS2 and rpL14 peptides is due to competition with ribosomes for binding to calmodulin, we performed additional *in vitro* translation assays in the presence of the putative CaM-binding peptide of rpL9, which was inferred not to bind to CaM (Figure 4 and Figure 5) and the known CaM inhibitor TFP. While TFP had an inhibitory effect on protein synthesis in line with earlier studies,[19] we observed no inhibition in the presence of rpL9 peptide (Figure S2, Supporting Information). These data show that rpS2 and rpL14 peptides inhibit protein synthesis in a competitive manner by binding to calmodulin. Additionally, we observed that the rpL14 and smMLCK peptides were much stronger inhibitors (IC_{50} values: 50 μM) than the rpS2 peptide (IC_{50} value: 500 μM) (Figure 6D). This suggests that under the conditions of the cell-free system, the rps2 peptide may bind to CaM either with lower affinity or that binding of this peptide results in a different conformational change of CaM compared to that in the rpL14 or smMLCK peptides. At high peptide concentrations (500 μM), the rpL14 peptide was the strongest inhibitor of protein synthesis (93% inhibition) followed by smMLCK (75% inhibition), while the rpS2 peptide showed only 52% inhibition of protein synthesis. At low peptide concentrations (5–10 μM), both rpL14 and rpS2 peptides

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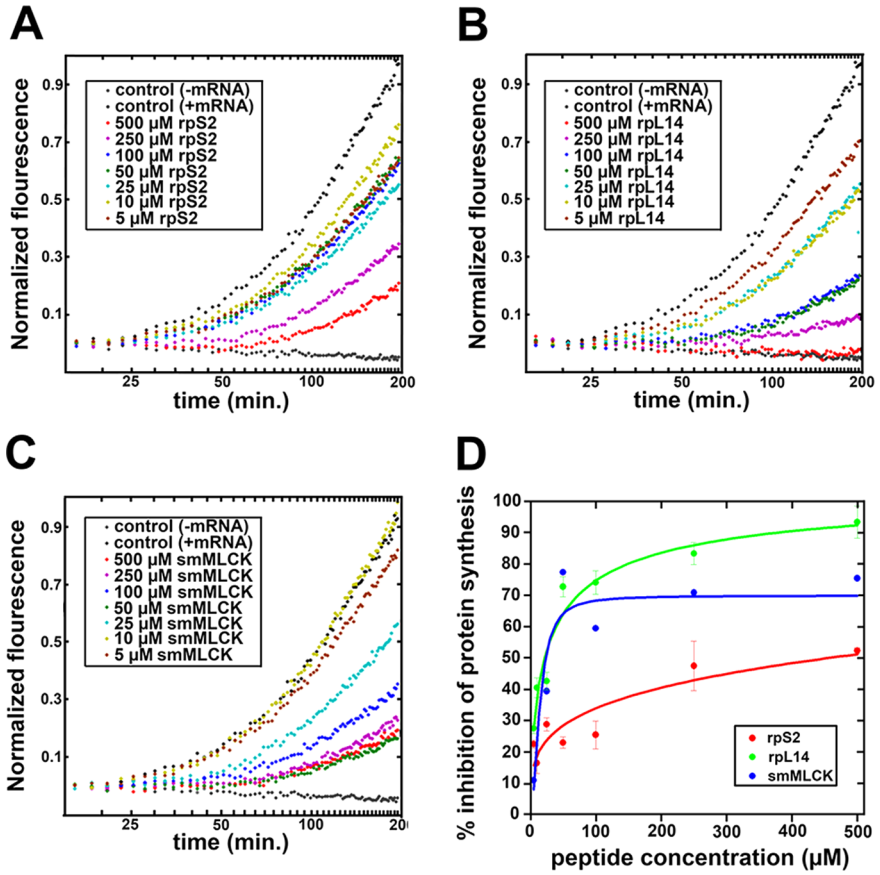

Figure 6. Inhibition of cell free translation with ribosomal CaM-binding peptides. Translational activity was measured using a Human *in Vitro* Protein Expression Kit (Pierce) and a GFP-mRNA as template. (A–C) The cell-free system was assayed for GFP fluorescence intensity for 200 min in the presence of different concentrations synthetic peptide from ribosomal protein S2 (A), L14 (B), or smMLCK (C). (D) Linear regression of the concentration-dependent inhibition of protein synthesis after 200 min in the presence of peptides revealed an IC_{50} value of 50 μM for rpL14 and smMLCK and an IC_{50} value of 500 μM for rpS2.

showed stronger inhibition potencies (22–30% inhibition) than that of the smMLCK peptide (≤10% inhibition) (Figure 6A–D). These data support the SPR competition data indicating that both ribosomal peptides seem to use different binding sites on CaM and/or trigger different conformational changes upon binding than smMLCK.

DISCUSSION

The process of protein translation is complex and tightly regulated, and one of many factors that play a role in its regulation are intracellular Ca^{2+}-signals.[52,13,18] As CaM is a ubiquitously expressed Ca^{2+}-binding protein that interacts with over 300 protein targets involved in a wide range of biological functions, it is no surprise that proteins involved in translation have been identified as putative CaM-targets. These targets include several free 40S and 60S ribosomal proteins[30−33] and ribosome-associated proteins like elongation factor-2, calmodulin kinases, fibroblast growth factor 3, peptidylarginine deiminase, and RNA-helicases.[34−41] This leads to the key questions of whether and how CaM is involved in protein translation. Evidence for a role of CaM in protein synthesis has been given by Kumar et al.[19] where they found that CaM antagonists inhibit the initiation of protein synthesis and that this inhibition is reversed by adding exogenous CaM. In the present study, we found that Ca^{2+}-CaM not only interacts with isolated ribosomal proteins as has been previously reported[31−33] but also with functional 80S ribosomes and polyribosomes (Figure 1A and 1B, and Figure 2A–C). This indicates that CaM interacts with ribosomes either directly through ribosomal proteins or through other ribosome-associated proteins. The failure of Mg^{2+} to replace Ca^{2+} to achieve high affinity binding suggests that the high specificity of the CaM–ribosome interaction relies on the Ca^{2+}-induced conformational change of CaM. We also found that CaM binds in a Ca^{2+}-dependent manner to polyribosomes (Figures 1B and 2D) indicating that Ca^{2+}-CaM also binds to actively translating ribosomal complexes. This result provides further evidence that CaM is involved in ribosomal activity. Synthetic peptides corresponding to the putative CaM-binding site of rpS2 and rpL14 interacted with CaM in a Ca^{2+}-dependent manner (Figure 4), like the intact 80S ribosome. Moreover, both peptides compete with ribosomes for binding to CaM (Figure 5) and have the ability to completely block CaM binding to ribosomes. Additionally, both ribosomal peptides, L14 and rpS2, inhibit protein synthesis with different potencies than smMLCK (Figure 6). This further supports that 80S ribosomes cannot bind to CaM when it is complexed with the ribosomal peptides.

Rps2 is located on the surface of the 40S subunit (Figure 3B) and is important for the function of the 40S subunit and in ribosome assembly. Mutations in rpS2 have been shown to affect translational fidelity.[53,54] Additionally, rpS2 interacts via an Arg-Gly-rich N-terminal region with fibroblast growth factor (FGF3), protein arginine methyltransferase 3 (PRMT3), and

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peptidylarginine deiminase (PAD).[55,56,39] PAD4 and PRMT3 citrullinate and methylate rpS2 antagonistically and have been found to cosediment with the free 40S subunit consistent with the findings that citrullination of rpS2 plays a role in the regulation of rpS2 and ribosome assembly.[39] In several CaM-regulated enzymes, the CaM-binding site is in close proximity to or overlapping with an autoinhibitory segment that blocks and down-regulates the enzyme in the absence of bound CaM.[57] The identified CaM-binding site of rpS2 is located apart from the Arg-Gly-rich N-terminal region in an extended loop buried in a pocket formed by 40S rRNA (Figure 3B). It is possible that the interaction of CaM with rpS2 influences the regulation of rpS2 by PRMT3, PAD, and/or FGF3 or that it influences the interactions with 40S rRNA and thereby regulates translational processes and/or ribosome assembly.

The ribosomal protein L14 is located on the surface of the 60S subunit (Figure 3B) and is involved in ribosomal 60S subunit biogenesis, rRNA processing, and translational elongation.[58−60] In *Drosophila*, reduced rpL14 gene expression leads to lethality and somatic anomalies in developing and differentiating cells.[61] Interaction of CaM with rpL14 might influence translational elongation and/or ribosomal biogenesis.

The findings described here provide strong evidence that CaM interacts directly with functional ribosomes and plays an important role in the Ca^{2+}-dependent regulation of protein synthesis. They also suggest many exciting directions for future research to understand the mechanisms, structure, and function of the interaction of CaM with ribosomes and its physiological relevance. Further investigations are needed to determine whether CaM binds to rpS2 and rpL14 in ribosomal complexes and whether it binds to other proteins in the ribosome as well, for example, other identified ribosomal CaM-binding proteins from the 40S (rpS8, rpS4, rpS15a, rpS14, and rpS20) and 60 subunits (rpL9, rpL22, and rpL13a).[30−32] Several studies have demonstrated that elevated Ca^{2+} levels modulate gene expression in plants.[62,63] It is also well-known that Ca^{2+} plays a central role in mechanisms of neuronal plasticity and is involved in the regulation of gene expression in neurons.[64] It will be a challenge to find out whether CaM-mediated regulation of protein synthesis is involved in processes of neuronal plasticity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Sucrose gradient centrifugation of polyribosomal fractions from HeLa cells, SPR analysis of CaM interactions with rRNA and polyrRNA, and translational activity without and with a peptide from the ribosomal protein L9 and the CaM inhibitor trifluoperazine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

CaM, calmodulin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FGF, fibroblast growth factor; GFP, green fluorescent protein; ITC, isothermal titration calorimetry; PAD, peptidylarginine deiminase; PAGE, polyacrylamid gelelectrophoresis; PRMT, protein arginine *N*-methyltransferase; rp, ribosomal protein; smMLCK, myosin light chain kinase; SPR, surface plasmon resonance

■ REFERENCES

(1) Wool, I. G., Chan, Y. L., and Glück, A. (1995) Structure and evolution of mammalian ribosomal proteins. Biochem. *Cell Biol. 73*, 933−947.

(2) Green, R., and Noller, H. F. (1997) Ribosomes and translation. *Annu. Rev. Biochem. 66*, 679−716.

(3) Ramakrishnan, V. (2002) Ribosome structure and the mechanism of translation. *Cell 108*, 557−572.

(4) Moldave, K. (1985) Eukaryotic protein synthesis. *Annu. Rev. Biochem. 54*, 1109−1149.

(5) Nygård, O., and Nilsson, L. (1990) Translational dynamics. Interactions between the translational factors, tRNA and ribosomes during eukaryotic protein synthesis. *Eur. J. Biochem. 191*, 1−17.

(6) Merrick, W. C. (1992) Mechanism and regulation of eukaryotic protein synthesis. *Microbiol. Rev. 56*, 291−315.

(7) Pestova, T. V., and Hellen, C. U. (2000) The structure and function of initiation factors in eukaryotic protein synthesis. *Cell Mol. Life. Sci. 57*, 651−674.

(8) Riis, B., Rattan, S. I., Clark, B. F., and Merrick, W. C. (1990) Eukaryotic protein elongation factors. *Trends Biochem. Sci. 15*, 420−424.

(9) Negrutskii, B. S., and El'skaya, A. V. (1998) Eukaryotic translation elongation factor 1 alpha: structure, expression, functions, and possible role in aminoacyl-tRNA channeling. *Prog. Nucleic Acid Res. Mol. Biol. 60*, 47−78.

(10) Czworkowski, J., and Moore, P. B. (1996) The elongation phase of protein synthesis. *Prog. Nucleic Acid Res. Mol. Biol. 54*, 293−332.

(11) Nakamura, Y., and Ito, K. (1998) How protein reads the stop codon and terminates translation. *Genes Cells 3*, 265−278.

(12) Kisselev, L. L., and Frolova, L. Y. (1999) Termination of translation in eukaryotes: new results and new hypotheses. *Biochemistry (Moscow) 64*, 8−16.

(13) Chin, K. V., Cade, C., Brostrom, C. O., Galuska, E. M., and Brostrom, M. A. (1987) Calcium-dependent regulation of protein synthesis at translational initiation in eukaryotic cells. *J. Biol. Chem. 262*, 16509−16514.

(14) Ledford, B. E., and Davis, D. F. (1978) Analysis of translational parameters in cultured cells. *Biochim. Biophys. Acta 519*, 204−212.

(15) Brostrom, C. O., Bocckino, S. B., and Brostrom, M. A. (1983) Identification of a Ca2+ requirement for protein synthesis in eukaryotic cells. *J. Biol. Chem. 258*, 14390−14399.

(16) Farese, R. V. (1971) Calcium as a mediator of adrenocorticotrophic hormone action on adrenal protein synthesis. *Science 173*, 447−450.

(17) Ruiz, N., and Krauskopf, M. (1980) Calcium stimulation of polyphenylalanine synthesis in a bovine heart cell-free system. *Life Sci. 27*, 2359−2365.

(18) Brostrom, C. O., and Brostrom, M. A. (1990) Calcium-dependent regulation of protein synthesis in intact mammalian cells. *Annu. Rev. Physiol. 52*, 577−590.

(19) Kumar, R. V., Panniers, R., Wolfman, A., and Henshaw, E. C. (1991) Inhibition of protein synthesis by antagonists of calmodulin in Ehrlich ascites tumor cells. *Eur. J. Biochem. 195*, 313−319.

(20) Van Eldik, L. J., and Watterson, D. M. (1998) *Calmodulin and Signal Transduction* (Van Eldik, L. J., and Watterson, D. M., Eds.) Academic Press, New York.
(21) Stull, J. T. (2001) Ca2+-dependent cell signaling through calmodulin-activated protein phosphatase and protein kinases, Minireview series. *J. Biol. Chem. 276*, 2311−2312.
(22) Vetter, S. W., and Leclerc, E. (2003) Novel aspects of calmodulin target recognition and activation. *Eur. J. Biochem. 270*, 404−414.
(23) Xia, Z., and Storm, D. R. (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat. Rev. Neurosci., 6*−267−276.
(24) Kahl, C. R., and Means, A. R. (2003) Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. *Endocr. Rev. 24*, 719−736.
(25) Clapham, D. E. (2007) Calcium signaling. *Cell*, 131−1047−1058.
(26) Hook, S. S., and Means, A. R. (2001) Ca(2+)/CaM-dependent kinases: from activation to function. *Annu. Rev. Pharmacol. Toxicol. 41*, 471−505.
(27) Klee, C. B., Ren, H., and Wang, X. (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem. 273*, 13367−13370.
(28) Weitz, D., Zoche, M., Müller, F., Beyermann, M., Körschen, H. G., Kaupp, U. B., and Koch, K. W. (1998) Calmodulin controls the rod photoreceptor CNG channel through an unconventional binding site in the N-terminus of the beta-subunit. *EMBO J. 17*, 2273−2284.
(29) Hathaway, D. R., Adelstein, R. S., and Klee, C. B. (1981) Interaction of calmodulin with myosin light chain kinase and cAMP-dependent protein kinase in bovine brain. *J. Biol. Chem. 256*, 8183−8189.
(30) Sonnemann, J., Bäuerle, A., Winckler, T., and Mutzel, R. (1991) A ribosomal calmodulin-binding protein from Dictyostelium. *J. Biol. Chem. 266*, 23091−23096.
(31) Shen, X., Valencia, C. A., Szostak, J. W., Dong, B., and Liu, R. (2005) Scanning the human proteome for calmodulin-binding proteins. *Proc. Natl. Acad. Sci. U.S.A. 102*, 5969−5974.
(32) Berggård, T., Arrigoni, G., Olsson, O., Fex, M., Linse, S., and James, P. (2006) 140 mouse brain proteins identified by Ca2+-calmodulin affinity chromatography and tandem mass spectrometry. *J. Proteome Res. 5*, 669−687.
(33) O'Connell, D. J., Bauer, M. C., O'Brien, J., Johnson, W. M., Divizio, C. A., O'Kane, S. L., Berggård, T., Merino, A., Åkerfeldt, K. S., Linse, S., and Cahill, D. J. (2010) Integrated protein array screening and high throughput validation of 70 novel neural calmodulin-binding proteins. *Mol. Cell. Proteomics 9*, 1118−1132.
(34) Kaul, G., Pattan, G., and Rafeequi, T. (2011) Eukaryotic elongation factor-2 (eEF2): its regulation and peptide chain elongation. *Cell Biochem. Funct. 29*, 227−234.
(35) Nairn, A. C., and Palfrey, H. C. (1987) Identification of the major Mr 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. *J. Biol. Chem. 262*, 17299−17303.
(36) Ryazanov, A. G. (1987) Ca2+/calmodulin-dependent phosphorylation of elongation factor 2. *FEBS Lett. 214*, 331−334.
(37) Maeda, N., Toku, S., Naito, Y., Nishiura, H., Tanaka, T., and Yamamoto, H. (2009) Phosphorylation of ribosomal protein S19 at Ser59 by CaM kinase I alpha. *J. Neurochem. 109*, 393−402.
(38) Mishra-Gorur, K., Singer, H. A., and Castellot, J. J., Jr. (2002) The S18 ribosomal protein is a putative substrate for Ca2+/calmodulin-activated protein kinase II. *J. Biol. Chem. 277*, 33537−33540.
(39) Guo, Q., Bedford, M. T., and Fast, W. (2011) Discovery of peptidylarginine deiminase-4 substrates by protein array: antagonistic citrullination and methylation of human ribosomal protein S2. *Mol. Biosyst. 7*, 2286−2295.
(40) Buelt, M. K., Glidden, B. J., and Storm, D. R. (1994) Regulation of p68 RNA helicase by calmodulin and protein kinase C. *J. Biol. Chem. 269*, 29367−29370.
(41) García-Gómez, J. J., Lebaron, S., Froment, C., Monsarrat, B., Henry, Y., and de la Cruz, J. (2011) Dynamics of the putative RNA helicase Spb4 during ribosome assembly in Saccharomyces cerevisiae. *Mol. Cell. Biol. 31*, 4156−4164.
(42) Belin, S., Hacot, S., Daudignon, L., Therizols, G., Pourpe, S., Mertani, H. C., Rosa-Calatrava, M., and Diaz, J. J. (2010) Purification of ribosomes from human cell lines. *Curr. Protoc. Cell. Biol.*, Chapter 3, Unit 3.40.
(43) Waltersson, Y., Linse, S., Brodin, P., and Grundström, T. (1993) Mutational effects on the cooperativity of Ca2+ binding in calmodulin. *Biochemistry 32*, 7866−7871.
(44) André, I., and Linse, S. (2002) Measurement of Ca2+-binding constants of proteins and presentation of the CaLigator software. *Anal. Biochem. 305*, 195−205.
(45) Dell'Orco, D., Xue, W. F., Thulin, E., and Linse, S. (2005) Electrostatic contributions to the kinetics and thermodynamics of protein assembly. *Biophys. J. 88*, 1991−2002.
(46) Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc. 1*, 2856−2860.
(47) Ben-Shem, A., Jenner, L., Yusupova, G., and Yusupov, M. (2010) Crystal structure of the eukaryotic ribosome. *Science 330*, 1203−1209.
(48) Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., and Ikura, M. (2000) Calmodulin target database. *J. Struct. Funct. Genomics 1*, 8−14.
(49) Marion, M. J., and Marion, C. (1988) Ribosomal proteins S2, S6, S10, S14, S15 and S25 are localized on the surface of mammalian 40 S subunits and stabilize their conformation. A study with immobilized trypsin. *FEBS Lett. 232*, 281−285.
(50) Spahn, C. M., Beckmann, R., Eswar, N., Penczek, P. A., Sali, A., Blobel, G., and Frank, J. (2001) Structure of the 80S ribosome from *Saccharomyces cerevisiae*–tRNA-ribosome and subunit-subunit interactions. *Cell 107*, 373−386.
(51) Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Target enzyme recognition by calmodulin: 2.4 A structure of a calmodulin-peptide complex. *Science 257*, 1251−1255.
(52) Kameyama, T., and Etlinger, J. D. (1979) Calcium-dependent regulation of protein synthesis and degradation in muscle. *Nature 279*, 344−246.
(53) Kruiswijk, T., de Hey, J. T., and Planta, R. J. (1978) Modification of yeast ribosomal proteins. Phosphorylation. *Biochem. J. 175*, 213−219.
(54) Bernstein, K. A., Gallagher, J. E., Mitchell, B. M., Granneman, S., and Baserga, S. J. (2004) The small-subunit processome is a ribosome assembly intermediate. *Eukaryot. Cell. 3*, 1619−1626.
(55) Antoine, M., Reimers, K., Wirz, W., Gressner, A. M., Müller, R., and Kiefer, P. (2005) Fibroblast growth factor 3, a protein with a dual subcellular fate, is interacting with human ribosomal protein S2. *Biochem. Biophys. Res. Commun. 338*, 1248−1255.
(56) Swiercz, R., Person, M. D., and Bedford, M. T. (2005) Ribosomal protein S2 is a substrate for mammalian PRMT3 (protein arginine methyltransferase 3). *Biochem. J. 386*, 85−91.
(57) James, P., Vorherr, T., and Carafoli, E. (1995) Calmodulin-binding domains: just two faced or multi-faceted? *Trends. Biochem. Sci. 20*, 38−42.
(58) Guo, Q., Bedford, M. T., and Fast, W. (2011) Discovery of peptidylarginine deiminase-4 substrates by protein array: antagonistic citrullination and methylation of human ribosomal protein S2. *Mol. Biosyst. 7*, 2286−2295.
(59) Robledo, S., Idol, R. A., Crimmins, D. L., Ladenson, J. H., Mason, P. J., and Bessler, M. (2008) The role of human ribosomal proteins in the maturation of rRNA and ribosome production. *RNA 14*, 1918−1929.
(60) Kapp, L. D., and Lorsch, J. R. (2004) The molecular mechanics of eukaryotic translation. *Annu. Rev. Biochem. 73*, 657−704.
(61) Enerly, E., Larsson, J., and Lambertsson, A. (2003) Silencing the Drosophila ribosomal protein L14 gene using targeted RNA interference causes distinct somatic anomalies. *Gene 320*, 41−48.

(62) van Der Luit, A. H., Olivari, C., Haley, A., Knight, M. R., and Trewavas, A. J. (1999) Distinct calcium signaling pathways regulate calmodulin gene expression in tobacco. *Plant Physiol. 121*, 705−714.
(63) Alizadeh, D., and Cohen, A. (2012) Red light and calmodulin regulate the expression of the psbA binding protein genes in *Chlamydomonas reinhardtii*. *Plant Cell. Physiol. 51*, 312−322.
(64) Ghosh, A., and Greenberg, M. E. (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science 268*, 239−247.