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A collection of caged compounds for probing roles of local translation in neurobiology

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ABSTRACT

Spatially localized translation plays a vital role in the normal functioning of neuronal systems and is widely believed to be involved in both learning and memory formation. It is of central interest to understand both the phenomenon and molecular mechanisms of local translation using new tools and approaches. Caged compounds can, in principle, be used as tools to investigate local translation since optical activation of bioactive molecules can achieve both spatial and temporal resolution on the micron scale and on the order of seconds or less, respectively. Successful caging of bioactive molecules requires the identification of key functional groups in appropriate molecules and the introduction of a suitable caging moiety. Here we present the design, synthesis and testing of a collection of three caged compounds: anisomycin caged with a diethylaminocoumarin moiety and dimethoxynitrobenzyl caged versions of 4E-BP and rapamycin. Whereas caged anisomycin can be used to control general translation, caged 4E-BP serves as a probe of cap-dependent translation initiation and caged rapamycin serves a probe of the role of mTORC1 in translation initiation. In vitro translation assays demonstrate that these caging strategies, in combination with the aforementioned compounds, are effective for optical control making it likely that such strategies can successfully employed in the study of local translation in living systems.

1. Introduction

Spatial and temporal control of protein synthesis (translation) plays a central role in the neural processes that underlie learning and memory (for reviews see Ref. 1–7). However, key questions remain unresolved, for example, does translation occur only in a stimulated dendritic spine or does it occur elsewhere, and are proteins targeted to that spine? In addition to establishing functional correlates of changes in local translation, one often wishes to probe the molecular mechanisms involved, that is, how does synaptic activity leading to long term potentiation (LTP) trigger translation of specific mRNAs in neurons? Is synthesis cap-dependent or is a cap-independent process involved?[8] Does signaling occur through the mTORC1 pathway (a central regulator of translation) or via other signaling cascades?[9] Answering such questions requires tools to dissect the regulation of local translation at a single synapse (length scale of ~1 μm) on the timescale of <1 min. Such tools could contribute substantially to the fundamental understanding of a variety of important neuronal processes.

Biochemical techniques for manipulating translation have been instrumental in establishing the connection between protein synthesis and development, learning and memory. In general, tools fall into two broad groups: (i) specific small molecule inhibitors and (ii) genetic approaches (e.g., overexpression and knock-outs). A variety of small molecules are known to inhibit eukaryotic translation. These include emetine, puromycin, anisomycin and cycloheximide. These inhibitors act primarily at the ribosome, preventing peptide bond formation, tRNA binding, or protein elongation;[10] however, additional small molecule inhibitors that other steps in protein synthesis, including translation initiation, are currently under development.[11–13] These small molecules are generally cell permeable but wash-in and wash-out are not very rapid and, although spatially restricted perfusion has been attempted,[14] these drugs are difficult to localize with confidence within micron sized areas.

Genetic approaches have also been used to alter translational efficiency by manipulating components of the protein-synthesis machinery.[15,16] While such approaches offer insights into the roles of specific proteins, as opposed to the all-or-none translational response in studies using general protein-synthesis inhibitors, they usually target global neuronal protein synthesis. With conditional control (e.g., using the CreER/loxP or DICE-K system) with cell type specific promoters, some degree of spatiotemporal control can be

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achieved as has been demonstrated in studies of hippocampal synaptic pathway function.[17]. Nevertheless, cell-based studies using these strategies may be difficult to interpret, particularly if the biological event under study is relatively fast (seconds/minutes) (e.g., LTP) or has a complex spatial dependence.

Photo-control, in which light is used to directly control a biochemical process, offers the possibility of rapid, spatially localized, external control. Caged compounds (compounds in which a light pulse is used to remove a protecting group and thereby render the molecule bioactive) already enjoy wide use in neurobiology.[18] Caged glutamate, in particular, has been used very successfully in studies of neural function including LTP.[19] In pioneering work, Dore, Schuman and colleagues designed and synthesized a caged version of anisomycin, a protein-synthesis inhibitor that functions at the level of the ribosome.[20]

We report here the design, synthesis, and in vitro evaluation of a collection of three new caged compounds that permit the photocontrol of different molecular steps involved in protein synthesis: (i) a diethylaminocoumarin-caged anisomycin derivative that extends the work of Goard et al.; (ii) a caged 4E-BP peptide that permits selective control of cap-dependent versus cap-independent modes of translation initiation and (iii) a caged rapamycin derivative that enables probing of the role of the mTORC1 pathway in regulating local translation.

2. Materials and methods

2.1. DEAC-caged anisomycin (*N*-([7-*N*,*N*-diethylaminocoumarin-4-yl]methyloxycarbonyl)anisomycin)

To a solution of 15 mg (0.06 mmol) of 7-(diethylamino)-4-(hydroxymethyl)-2*H*-chromen-2-one (prepared exactly as described,[21]) in dry acetonitrile was added 12 mg (0.06 mmol) 4-nitrophenyl chloroformate and the solution was stirred for 7 h at room temperature under argon. Then 10 mg (0.08 mmol) of 4-dimethylaminopyridine and 10 mg (0.038 mmol) of anisomycin (AG Scientific) was added and the solution was stirred overnight. The reaction was quenched with 3–4 vol 15% citric acid, and extracted with chloroform. The product was purified by HPLC using a Zorbax SB-C18 column (Chromatographic Specialties Inc.) using isocratic methanol/water 70/30 conditions with a flow rate of 5 mL/min. The product eluted at 13.4 min. ^{1}H NMR (400 MHz, CD_3OD, 1.3:1 mixture of conformational isomers) δ 7.41 (d, J = 9.0 Hz, 1H), 7.00 and 6.89 (d, J = 8.0 Hz, 2H), 6.72 (d, J = 8.0 Hz, 2H), 6.67–6.60 (m, 2H), 6.46 (s, 1H), 5.98 and 5.92 (s, 2H), 5.26 and 5.13 (AB q, 2H), 4.35–4.28 (m, 1H), 4.02 and 3.90 (br s, 1H), 3.65 and 3.60 (s, 3H), 3.50–3.47 (m, 1H), 3.39 (q, J = 7.0 Hz, 4H), 3.25–3.08 (m, 1H), 2.92–2.87 (m, 1H), 2.70 (t, J = 13.2 Hz, 1H), 1.99 and 1.95 (s, 3H), 1.09 (t, J = 7.0 Hz, 6H); ESI-HRMS: ($C_{29}H_{35}N_2O_8$)(MH^+), calcd 539.2387, found 539.2396. Uncaging was tested by exposing DEAC-caged anisomycin solutions in 50 mM HEPES buffer pH 7.0 with 5% methanol for solubility to light from a 405 nm high intensity LED (Super Bright LEDs Inc. St. Louis Missouri, Model RL5-UV2030 (405 nm; ~20 mW/cm^2)). Disappearance of caged anisomycin and appearance of anisomycin were followed by HPLC (Zorbax SB-C18 column acetonitrile/water 60/40 (+0.1% trifluoroacetic acid) for caged anisomycin (eluted at 22 min (detect at 380 nm); acetonitrile/water 35/65 (+0.1% trifluoroacetic acid) for anisomycin (eluted at 9.3 min (detect at 225 nm)) and ESI-HRMS: ($C_{14}H_{20}NO_4$)(MH^+), calcd 266.1386, found 266.1393.

3. 4E-BP peptides

FMOC protected amino acids, Rink amide resin and HBTU were obtained from Anaspec Inc. Preparation of FMOC-Tyr(*O*-(4,5-dimethoxy-2-nitrobenzyl))-OH was carried out as described previously.[22] Dimethoxy-2-nitrobenzyl bromide was prepared from veratraldehyde as described.[23,24] Peptides were prepared using standard Fmoc-based solid-phase peptide synthesis in a microwave peptide synthesizer (CEM Corp.). After synthesis, peptides were HPLC purified using a Zorbax SB-C18 column (Chromatographic Specialties Inc.) using a linear gradient of 5–60% acetonitrile/water containing 0.1% trifluoroacetic acid in 20 min was used with a flow rate of 2 mL/min. Peptide composition was confirmed by ESI-MS. 4E-BP1a caged: PGGARIIY(dmnb)DRKFLMAib-AibRNAPVAKAPP; calcd: 3003.8, found: 3002.7. Uncaging was tested by exposing peptide solutions in water to light from a 365 nm high intensity LED (Opto Technology, Inc., Wheeling, IL, Model OTLH-0480-UV (365 nm, ~20 mW/cm^2) and following conversion using ESI-MS (uncaged PGGARIIYDRKFLMAibAibRNAPVA KAPP) calcd: 2808.34, found: 2807.6.

3.1. Caged rapamycin (7-demethoxy-7-dimethoxy-2-nitrobenzyloxy-rapamycin)

Rapamycin (LC Laboratories, Woburn, MA) (100 mg, 0.110 mmol) was dissolved in dry dichloromethane (3 mL) at −40 °C under argon then 100 μL trifluoroacetic acid was added and the solution was stirred for 10 min essentially as described.[25] Ten equivalents of dimethoxy-2-nitrobenzyl alcohol (prepared from veratraldehyde as described[23,24]) were added and the solution stirred for 1 h at −40 °C. The mixture was then extracted with 5% aqueous sodium bicarbonate and dried over anhydrous sodium sulfate. The product was purified by preparative HPLC using a Zorbax SB-C18 column 9.4 × 25 cm (Chromatographic Specialties Inc.) using isocratic methanol/water 85/15 conditions with 0.1% acetic acid and a flow rate of 5 mL/min (eluted 10 min) followed by a second HPLC step using acetonitrile/water 80/20 Zorbax SB-C18 4.5 × 25 cm 2 mL/min (eluted at 4.5 min) (yield 11 mg, ~10%). ^{1}H NMR ($CDCl_3$, 400 MHz) (mixture of trans/cis conformational isomers; data for major isomer) δ 7.71 (s, 1H, DMNB Ar H-6′), 7.54 (s, 1H, DMNB Ar H-3′), 6.39–6.32 (m, 1H), 6.22–6.09 (m, 2H), 6.02–6.00 (m, 1H), 5.60–5.50 (m, 1H), 5.50–5.35 (m, 2H), 5.35–5.27 (m, 1H), 5.25–5.1 (m, 1H), 4.72 (s, 1H, OH-13), 4.35–4.25 (m, 2H (CH_2 on DMNB)), 4.18 (1H, H-28, indicating C7-*S* epimer), 3.99 (s, 3H, CH_3O on DMNB), 3.94 (s, 3H, CH_3O on DMNB), 3.90–3.85 (m, 1H), 3.7 (s, 1H, H-29), 3.7 (m, 1H), 3.41 (s, 3H), 3.34 (s, 3H), 2.94 (m, 1H), 2.64 (d, 2H), 1.75 (s, 3H), 1.40–0.83 (set of broad unresolved peaks, similar to rapamycin) (note singlet at 3.14 ppm (C7-methoxy in rapamycin is not present as expected)). ESI-HRMS: ($C_{59}H_{86}N_2O_{17}Na$)(MNa^+), calcd 1117.5819, found 1117.5822. UV–vis (MeOH) λ_{max} 263, 274, 287, 350 nm. Uncaging was tested by exposing DMNB-caged rapamycin solutions in water with 50% methanol for solubility to light from a 365 nm high intensity LED (Opto Technology, Inc., Wheeling, IL, Model OTLH-0480-UV (365 nm, ~20 mW/cm^2)). Disappearance of caged rapamycin and appearance of demethyl-rapamcyin (i.e., rapamycin with OH at C7) were followed by HPLC (Zorbax SB-C18 acetonitrile/water 70/30 3.5 mL/min, detected at 254 nm) and ESI (demethyl-rapamycin) ESI-MS: ($C_{50}H_{77}NO_{13}Na$)(MNa^+), calcd: 922.5287, found: 922.5293. UV–vis (MeOH) λ_{max} 266, 275, 288.

3.2. DNA/RNA preparation

The bicistronic plasmid pSP-$(CAG)_{33}$-FF-HCV-Ren-p$(A)_{51}$ was a kind gift from Jerry Pelletier.[12,11] The plasmid was linearized by digestion with BamHI. The transcription reaction contained ribonucleotides from NEB (0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP), 0.5 mM $m^7G(5')ppp(5')G$ (Ambion), 100 U RNase inhibitor (BioShop), 10 μL 10× transcription buffer, 150 U SP6 polymerase (NEB), 3 μg linearized pSP-$(CAG)_{33}$-FF-HCV-Ren-

$p(A)_{51}$ and water to 100 μL. The reaction was incubated at 40 °C for 2 h, following which 6 μL of RNase-free DNase was added and the mixture incubated a further 15 min at 37 °C. For coupled transcription/translation reactions in rabbit reticulocyte lysate control Firefly luciferase DNA was used as supplied by the manufacturer.

3.3. In vitro translation reactions

In vitro translations in rabbit reticulocyte lysate were performed according to manufacturer's recommendations (Promega). In vitro translations in Krebs extracts were preformed as described previously.[11,12,26] Translation reactions contained 7 μL Krebs-2 cell extract, 50 ng RNA, 0.625–10 μg 4E-BP peptide and were made up to a final volume of 10 μL with 25 mM Hepes, pH 7.5. Reactions were incubated at 30 °C for 1 h and terminated with the addition of 20 μL of ice-cold PBS. Three microliters of reaction mix were added to 25 μL luciferase reagent from the Dual-Luciferase® reporter system (Promega) to quantify firefly luciferase translation and 25 μL of Stop & Glo® Reagent to quantify Renilla luciferase.

3.4. HeLa cell studies

Cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 100 units/mL gentamicin at 37 °C and 5% CO_2 in 75-cm^2 flasks. Cells were passaged by 0.25% Trypsin–EDTA when they reached ~80% confluence. Rapamycin and caged rapamycin stock solution were prepared in MeOH/water 50/50 then diluted in water so that volumes of 20–50 μL of each sample was added to the cell culture well (final concentration 20–50 nM). For uncaging experiments samples were irradiated for 1 min using a 365 nm LED (Opto Technology, Inc., Wheeling, IL, Model OTLH-0480-UV (365 nm, ~20 mW)). Cells were then incubated with drug for 1 h then washed with 1 mL PBS followed by 0.3 mL SDS gel loading buffer. This procedure caused detaching of the cells as a slurry, which was then boiled for 6 min and frozen. Samples (30 μL) were then applied to a 10% SDS–polyacrylamide gel, then transferred to a nitrocellulose membrane (Protran, Perkin–Elmer). Membranes were blocked in 5% nonfat milk in TBS containing 0.1% Tween 20 and probed with the appropriate primary and secondary antibodies (phospho-S6K1 (Thr389), from Cell Signaling, phospho-S6 (Ser240/244) also from Cell Signaling, and beta-actin, from Sigma) at 4 °C overnight. Immunoreactive proteins were visualized using enhanced chemiluminescence (Perkin–Elmer).

4. Results and discussion

4.1. DEAC-caged anisomycin

Anisomycin (**1**) is a cell permeable natural product that inhibits protein synthesis by binding to the peptidyl transferase site of the ribosome.[27,28] Atomic resolution structures of the antibiotic in complex with the large ribosomal subunit indicate that bulky substituents on the amino group of anisomycin will severely inhibit binding.[28] Dore, Schumann and colleagues reported anisomycin derivatives in which the amino group was caged with either dimethoxynitrobenzyl (DMNB) (**2**) or bromohydroxycoumarin (BHC) (**3**) derivatives. Both compounds were shown to be effective caged inhibitors of protein synthesis with uncaging at 365 nm (λ_{max} are 350 and 373 nm, respectively). Here we extend these studies by replacing the BHC group with diethylaminocoumarin moiety (**4**). While undergoing analogous photochemistry to BHC, the DEAC group has longer wavelength absorption (λ_{max} 395 with significant absorbance to 420 nm, compared to λ_{max} 373 nm for BHC) and a significantly higher molar extinction coefficient (~20,000 vs 14,500 M^{-1} cm^{-1}). In addition, quantum yields for uncaging as high as 0.3 have been reported.[21,29–31] Because an integrated 405 nm laser line that can be used for uncaging is becoming a common feature of a range of optical microscopes used for neuroimaging,[32,33] a caged anisomycin derivative that could be activated effectively with this light source may prove practically useful (Fig. 1).

DEAC-caged coumarin (**4**) was synthesized via oxidation of 7-(diethylamino)-4-methyl-2*H*-chromen-2-one with selenium dioxide as described[21] followed by coupling to anisomycin using *p*-nitrophenylchloroformate. Uncaging was tested using an HPLC assay (see Section 2) and using an in vitro translation assay. Translation was performed using rabbit reticulocyte lysate coupled transcription translation system using firefly luciferase DNA as template. Translational activity is measured by the amount of luciferase produced, which can be assayed accurately as relative light units produced under a standard set of conditions. Caged anisomycin was either uncaged immediately prior to adding to lysate or uncaged in situ with a 405 nm LED (20 mW/cm^2 radiant flux). Absorbance by the hemoglobin in the lysate resulted in longer half lives for uncaging in situ. The transcription/translation reactions were incubated for a further 1 h at 30 °C after uncaging before assaying for luciferase activity. Very large changes (three orders of magnitude) in translation rates were observed upon uncaging DEAC-anisomycin (Fig. 2).

Effective applied concentrations and light exposure times for use in living cells will require optimization in specific systems since maximal localization of the effect will require release of sufficient anisomycin to cause inhibition but not enough that diffusion leads to inhibitory concentrations outside the zone of interest. DEAC-caged anisomycin provides an easy-to-use starting point for probing the role of local translation in the nervous system.

4.2. Caged 4E-BP peptide for the inhibition of cap-dependent translation initiation

In most cases translation initiation is the rate limiting step in protein synthesis.[34] Translation initiation in eukaryotes can be

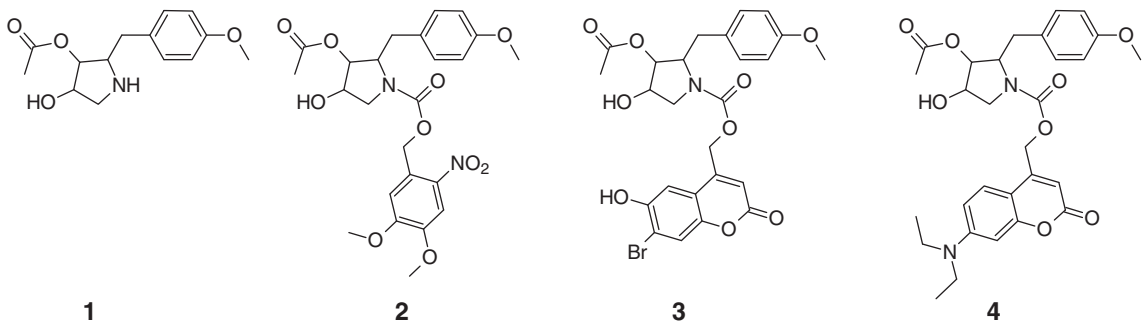

Figure 1. Structures of anisomycin (**1**), DMNB-caged anisomycin (**2**), BHC-caged anisomycin (**3**) and DEAC-caged anisomycin (**4**).

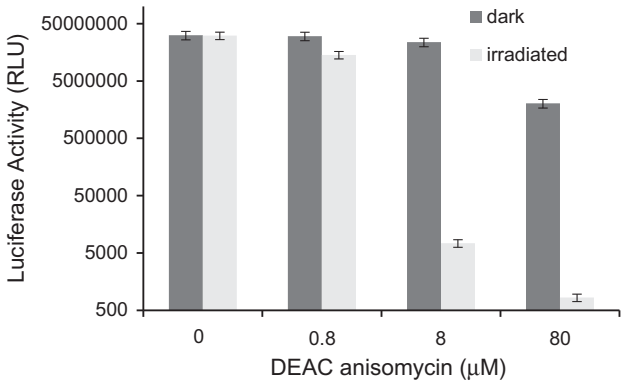

Figure 2. Measured luciferase activity (relative light units) (note log scale). The final concentration of caged anisomycin in the translation assays was 80, 8 and 0.8 μM. Dark gray bars are caged, light gray bars are uncaged (after exposure to a 405 nm LED for 10 s). The 8 μM concentration provides three orders of magnitude greater inhibition after uncaging.

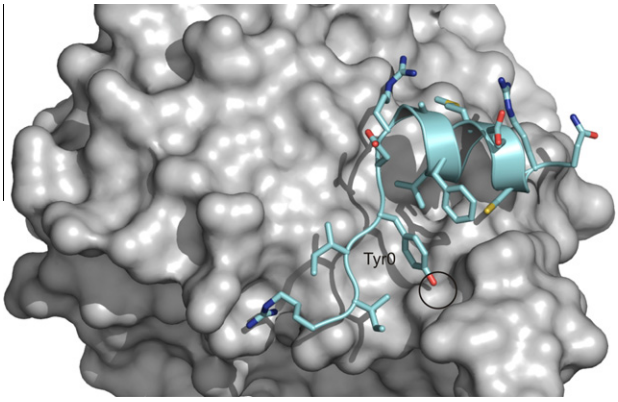

Figure 3. Structure of the 4E-BP1 peptide (cyan) bound to the eIF4E protein (gray surface). The essential Tyr(0) is labeled and the site of attachment for the caging group circled. The image is based on the X-ray data of Marcotrigiano et al.[48] (PDB code 1EJ4). Figure prepared using Pymol.

cap-dependent requiring the presence of the m^7GpppN cap structure (where 'm' represents a methyl group and 'N' is any nucleotide) at the 5′ end of the messenger (m)RNA. Conversely, in specific instances ribosomes can assemble at an IRES (internal ribosome entry site) sequence in the mRNA[36–38] to initiate cap-independent translation. Local control of the mode of translation initiation has been proposed to play a role in neurohormone secretion,[8] amyloid precursor protein production,[39] and FGF-2 production during brain development.[40]

The primary mechanism for regulating cap-dependent translation initiation is via binding of the 4E-BP proteins to the cap-binding translation initiation factor eIF4E. Three 4E-BPs have been identified (4E-BP1, 4E-BP2, 4E-BP3) together with the related proteins cup, maskin and neuroguidin.[41–44] These proteins appear to act as competitive inhibitors of eIF4G binding to eIF4E.[41,42] All 4E-BPs share with eIF4G a consensus eIF4E binding sequence. The sequence is shown below, numbered from the consensus Tyr at position zero. The symbols: b, X, f, φ and PO4 represent partially conserved hydrophobic, any residue, partially conserved phenylalanine, conserved hydrophobic and phosphorylation site, respectively.

Peptides corresponding to eIF4E binding sequences from 4E-BP1, 4E-BP2, and eIF4G have been shown to directly interact with purified recombinant eIF4E in vitro[13,45] with affinities ranging from 10 to 100 nM. These peptides have also been reported to inhibit in vitro translation in cell lysates.[46] The effectiveness of 4E-BP1 peptides as translation initiation inhibitors in vivo has also been tested by microinjection of peptides into sea urchin embryos where dramatic effects on maturation were observed consistent with peptide inhibition of cap-dependent translation initiation.[47]

An effective means for developing photo-control of cap-dependent translation initiation would therefore be to develop a caged 4E-BP analogue. Although small molecule inhibitors of the 4E/4G interaction are under development,[12,13] the affinity and selectivity of these does not yet match the native peptide sequences. For cell-based studies of local translation where photochemical tools will be applied locally over relatively short time scales, there is no requirement for tools to withstand the gut or the bloodstream, factors that limit the use of peptides broadly in drug development.

Substitution of the conserved Tyr (Y0) and Leu residues (L5) is known to severely inhibit binding of 4E-BPs to eIF4E.[45] This effect can be understood in structural terms because X-ray crystal data show both conserved residues make extensive contacts with the eIF4E protein.[48,49] Figure 3 shows a close up view of the interactions between Tyr0 and eIF4E. Caging this Tyr residue is expected to lead to severe steric clashes between the peptide and the protein, so that peptide binding is prevented.

We prepared FMOC-Tyr-DMNB and incorporated it into an extended 4E-BP peptide analogue (caged 4E-BP, Table 1). Starting with the 4E-BP1 sequence (for which a K_d of <50 nM has been measured with purified eIF4E[48]) we replaced the GluCys dipeptide sequence with AibAib and we removed potential phosphorylation sites (Ser11, Thr14). The AibAib unit promotes helical backbone conformations[50] thereby preorganizing the peptide into the bound conformation to enhance affinity. Removing phosphorylation sites eliminates the possibility that inhibitory effects of the 4E-BP analogues will be modified by the activity of kinases or phosphatases.

To test the ability of the caged 4E-BP peptide to photo-control cap-dependent translation, we prepared bicistronic mRNAs containing a capped transcript for firefly luciferase together with a Renilla luciferase transcript under control of an IRES sequences[12] (Fig. 4). Since firefly and Renilla luciferase enzymes use different substrates, they can be assayed together. Comparing the ratio of firefly luciferase to Renilla luciferase activity provides a direct measure of the relative rates of cap-dependent versus cap-independent (IRES mediated) translation in vitro.[12] We carried out in vitro translation reactions in Krebs ascites lysates using this bicistronic RNA in the presence of different amount of 4E-BP peptides. The Krebs lysate has been found to more faithfully reproduce the cap-dependent translation initiation process as compared to the rabbit reticulocyte system.[26]

Figure 5 shows results obtained using in vitro translations in Krebs lysates. Irradiation with 365 nm light for 30 s produced un-

Table 1
Consensus 4E binding sequence and 4E-BP1

Position					−3	−2	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14			
Consensus					R/K	b	X	**Y**	X	X	X	f	L	φ	X	X	X	X	PO4	X	X	X			
4E-BP1					R	I	I	**Y**	D	R	K	F	L	M	E	C	R	N	S	P	V	T			
Caged-4E-BP	P	G	G	A	R	I	I	Y	D	R	K	F	L	M	Aib	Aib	R	N	A	P	V	A	K	A	PP

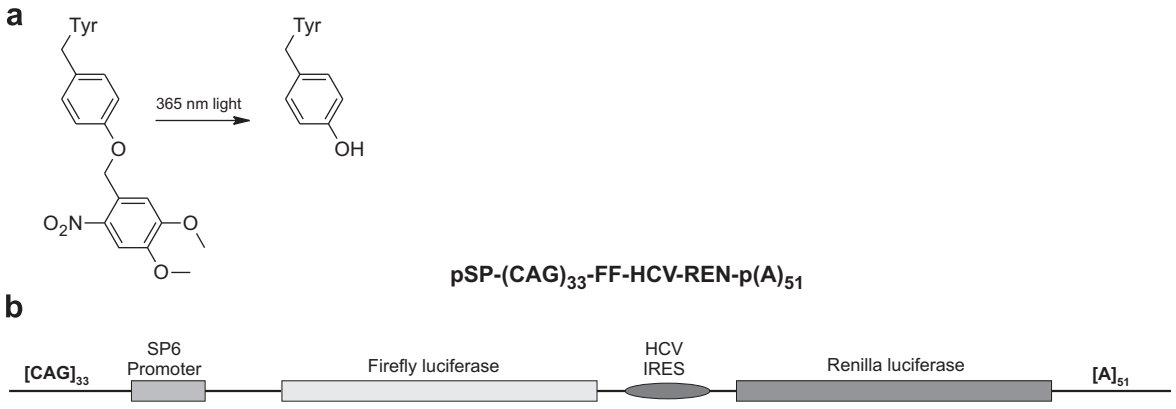

Figure 4. (a) Structure of DMNB-caged Tyr, (b) structure of bicistronic construct for analysis of cap-dependent translation.

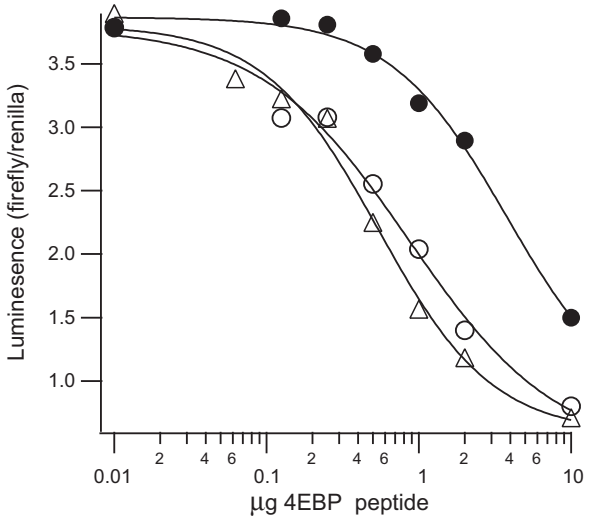

Figure 5. Inhibition of cap-dependent translation as measured by the ratio of firefly to Renilla luciferase activity in Krebs ascites extracts. Increasing amounts of caged (●), irradiated (caged, irradiated for 60 s) (○), and native 4E-BP peptides (△) were added to the reactions.

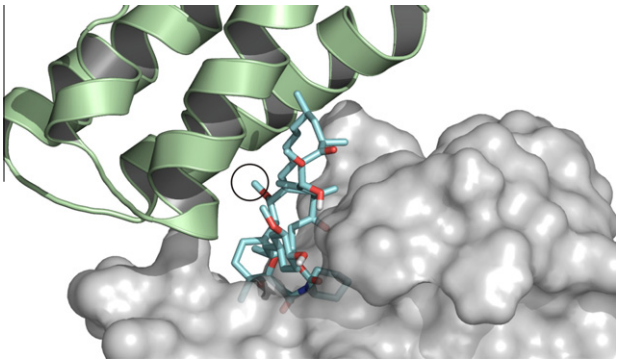

Figure 6. Rapamycin/FKBP/mTORC1 ternary complex. The 7-methoxy substituent of rapamycin (circled) points directly at mTORC1 (ribbon).

caged 4E-BP peptide which showed identical activity to pure synthetic 4E-BP peptide (Fig. 5) and more than an order of magnitude stronger inhibition of cap-dependent translation than the caged peptide. This result demonstrates that the Tyr0 site is indeed an effective site for caging. Even more effective caging may be obtained with a bulkier caging group such as a coumarin derivative[51] or the nitrodibenzofuran compound recently reported by Momotake et al.[52]

In order to be used for probing the role for cap-dependent versus cap-independent inhibition of translation in a cellular setting, caged 4E-BP peptides would have to be microinjected[47] or loaded via cell-uptake sequences. A variety of such sequences can be appended to the peptide during synthesis. Indeed Herbert et al. have reported 4E-BP peptides bearing penetrating cell-uptake sequences to be active in human lung fibroblast cells in culture.[45]

4.3. Caged rapamycin for the inhibition of mTORC1

4E-BP activity is post-translationally regulated via phosphorylation.[53] Upstream of 4E-BP is the central regulatory kinase mTORC1.[42] mTORC1 integrates the input from multiple pathways such as those triggered by insulin and growth factors.[54] mTORC1 phosphorylates S6K1 and this stimulates the subsequent phosphorylation of S6K1 by PDK1. Active S6K1 can in turn stimulate the initiation of protein synthesis through activation of S6 ribosomal protein (a component of the ribosome) and other components of the translational machinery.[55] Translation may also be regulated in other ways including pathways involving ERK and p38 MAPK signaling.[56,57] For instance, ARC synthesis during LTP consolidation in the dentate gyrus appears to be controlled via ERK-MNK signaling rather than via mTORC1.[9] A tool for the local control of mTORC1 would enable determination of the role of mTORC1 in local translation in variety of settings.mTORC1 is known to be specifically inhibited by the membrane-permeable natural product rapamycin.[58] Rapamycin acts by binding to FKBP (K_d = 0.5 nM) and this complex binds to and inhibits mTORC1.[25] A large medicinal chemistry patent literature describes numerous chemical modifications of rapamycin and their activities. One face of the rapamycin molecule interacts with FKBP and another face (called the effector domain) interacts with mTORC1.[58,59] Detailed structural information is available for complexes of rapamycin with FKBP and for the ternary complex FKBP–rapamycin–mTOR.[59,60] An examination of the X-ray crystal structure of the complex of FKBP/rapamycin with mTOR shows the position of the C7-methoxy group of rapamycin directly exposed to the mTOR interface (Fig. 6). Thus, introduction of a bulky caging group here is expected to effectively block mTORC1 activation.

Luengo et al. discovered that the methoxy position at C7 could be selectively substituted in a mild acid catalyzed process.[25] This remarkable transformation presumably results from the participation of the adjacent triene unit. Using this acid catalyzed substitution reaction we introduced a DMNB group at the C7 position of rapamycin (Fig. 7). We opted to use the DMNB group since (i)

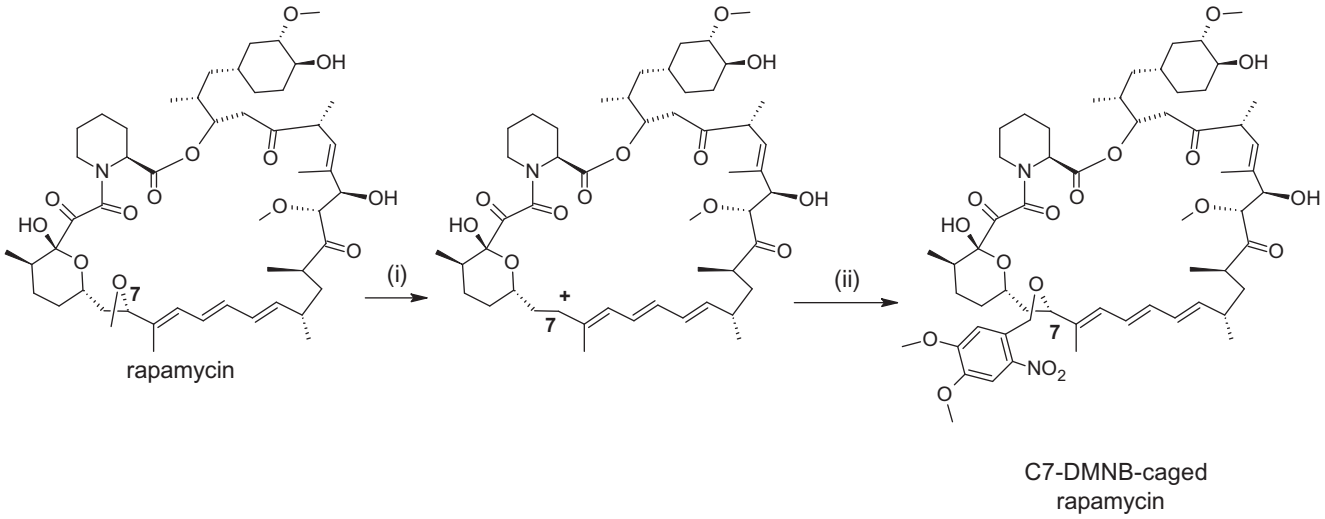

Figure 7. Synthesis of DMNB-caged rapamycin. (i) Trifluoroacetic acid in dichloromethane, (ii) DMNB alcohol.

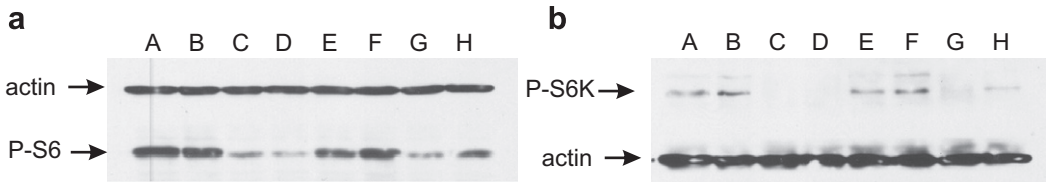

Figure 8. Western blots for phospho-S6 (a) and phosphor-S6K (b) (actin serves as an internal control for total protein concentration). A: control, B: vehicle, C: 50 nM rapamycin, D: 20 nM rapamycin, E: 50 nM caged rapamycin, F: 20 nM caged rapamycin, G: 50 nM uncaged (+UV) rapamycin, H: 20 nM uncaged (+UV).

the dimethoxynitrobenzyl alcohol undergoes the acid catalyzed substitution reaction easily, (ii) it provides sufficient steric bulk for caging and (iii) it undergoes uncaging at a wavelength (365 nm) that does not overlap significantly with the triene unit or rapamycin (near 280 nm). Uncaging is expected to produce 7-hydroxy rapamycin rather than native rapamycin. This compound has been reported to be active,[61] presumably because it retains the favorable interactions of rapamycin with its protein partners and does not introduce any steric hindrance.

Cell-based activity assays of this DMNB-caged rapamycin analogue were carried out using established assays.[62,63] HeLa cells were incubated with either vehicle, native rapamycin, DMNB-caged rapamycin or UV irradiated DMNB-caged rapamycin. Inhibition of mTORC1 was assayed by probing for changes in S6 and S6K phosphorylation levels via Western blot (Fig. 8). Caging was observed to block rapamycin activity very effectively since >10-fold differences in phospho-S6 were observed (Fig. 8a, lane D vs F). Importantly, near native levels of activity were restored upon uncaging indicating that DMNB-caged rapamycin behaves as designed.

An NBDF caged rapamycin could be produced by an analogous synthetic route. The larger size of the NBDF group may lead to even great differences between caged and uncaged derivatives. In addition, the NDBF group is expected to show better two-photon uncaging properties than the DMNB-caged rapamycin.[52] While coumarin derivatives such as diethylaminocoumarin are appealing for longer wavelength uncaging, they have been reported to be ineffective for caging alcohols directly.[29] Instead a 7-hydroxy rapamycin derivative would have to be caged as a coumarin carbonate. Alternatively, Wosnick et al. have reported that coumarins may be used to cage thiols directly.[64] Thus, a caged C7 mercapto rapamycin may be envisaged. All these derivatives are expected to be membrane permeable based on their similarity to numerous other rapamycin derivatives.[58] We therefore anticipate that they will be of direct use in experiments aimed at uncovering the role of mTORC1 in local translation.

5. Summary

We have reported the successful caging of three compounds, anisomycin, 4E-BP and rapamycin, with well-defined roles in the molecular mechanisms of translation. The sites for caging and the choice of caging group lead in each case to a compound that shows a substantial (10–1000 times) difference in activity upon optical activation. These compounds or related derivatives, when optimized for particular applications may prove useful for probing the roles and mechanisms of local translation in the nervous systems of animal models.

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References and notes

1. Bramham, C. R.; Wells, D. G. *Nat. Rev. Neurosci.* **2007**, *8*, 776.
2. Pfeiffer, B. E.; Huber, K. M. *J. Neurosci.* **2006**, *26*, 7147.
3. Schuman, E. M.; Dynes, J. L.; Steward, O. *J. Neurosci.* **2006**, *26*, 7143.
4. Banko, J. L.; Klann, E. *Prog. Brain Res.* **2008**, *169*, 59.
5. Klann, E.; Dever, T. E. *Nat. Rev. Neurosci.* **2004**, *5*, 931.
6. Govindarajan, A.; Kelleher, R. J.; Tonegawa, S. *Nat. Rev. Neurosci.* **2006**, *7*, 575.
7. Bourne, J.; Harris, K. M. *Curr. Opin. Neurobiol.* **2007**, *17*, 381.
8. Wayne, N. L.; Lee, W.; Michel, S.; Dyer, J.; Sossin, W. S. *Biol. Reprod.* **2004**, *70*, 277.
9. Panja, D.; Dagyte, G.; Bidinosti, M.; Wibrand, K.; Kristiansen, A. M.; Sonenberg, N.; Bramham, C. R. *J. Biol. Chem.* **2009**, *284*, 31498.
10. Janzen, D. M.; Geballe, A. P. *Cold Spring Harb. Symp. Quant. Biol.* **2001**, *66*, 459.

11. Novac, O.; Guenier, A. S.; Pelletier, J. *Nucleic Acids Res.* **2004**, *32*, 902.
12. Cencic, R.; Robert, F.; Pelletier, J. *Methods Enzymol.* **2007**, *431*, 269.
13. Moerke, N. J.; Aktas, H.; Chen, H.; Cantel, S.; Reibarkh, M. Y.; Fahmy, A.; Gross, J. D.; Degterev, A.; Yuan, J.; Chorev, M.; Halperin, J. A.; Wagner, G. *Cell* **2007**, *128*, 257.
14. Sutton, M. A.; Ito, H. T.; Cressy, P.; Kempf, C.; Woo, J. C.; Schuman, E. M. *Cell* **2006**, *125*, 785.
15. Banko, J. L.; Hou, L.; Poulin, F.; Sonenberg, N.; Klann, E. *J. Neurosci.* **2006**, *26*, 2167.
16. Costa-Mattioli, M.; Gobert, D.; Harding, H.; Herdy, B.; Azzi, M.; Bruno, M.; Bidinosti, M.; Ben Mamou, C.; Marcinkiewicz, E.; Yoshida, M.; Imataka, H.; Cuello, A. C.; Seidah, N.; Sossin, W.; Lacaille, J. C.; Ron, D.; Nader, K.; Sonenberg, N. *Nature* **2005**, *436*, 1166.
17. Nakashiba, T.; Young, J. Z.; McHugh, T. J.; Buhl, D. L.; Tonegawa, S. *Science* **2008**, *319*, 1260.
18. Ellis-Davies, G. C. *Nat. Methods* **2007**, *4*, 619.
19. Tanaka, J.; Horiike, Y.; Matsuzaki, M.; Miyazaki, T.; Ellis-Davies, G. C.; Kasai, H. *Science* **2008**, *319*, 1683.
20. Goard, M.; Aakalu, G.; Fedoryak, O. D.; Quinonez, C.; St Julien, J.; Poteet, S. J.; Schuman, E. M.; Dore, T. M. *Chem. Biol.* **2005**, *12*, 685.
21. Shembekar, V. R.; Chen, Y.; Carpenter, B. K.; Hess, G. P. *Biochemistry* **2005**, *44*, 7107.
22. Wang, Q.; Dai, Z.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 14016.
23. Fletscher, C. A. *Org. Synth.* **1953**, *33*, 65.
24. Wilcox, M.; Viola, R. W.; Johnson, K. W.; Billington, A. P.; Carpenter, B. K.; Mccray, J. A.; Guzikowski, A. P.; Hess, G. P. *J. Org. Chem.* **1990**, *55*, 1585.
25. Luengo, J. I.; Yamashita, D. S.; Dunnington, D.; Beck, A. K.; Rozamus, L. W.; Yen, H. K.; Bossard, M. J.; Levy, M. A.; Hand, A.; Newman-Tarr, T., et al *Chem. Biol.* **1995**, *2*, 471.
26. Svitkin, Y. V.; Sonenberg, N. *Methods Enzymol.* **2007**, *429*, 53.
27. Hansen, J. L.; Moore, P. B.; Steitz, T. A. *J. Mol. Biol.* **2003**, *330*, 1061.
28. Blaha, G.; Gurel, G.; Schroeder, S. J.; Moore, P. B.; Steitz, T. A. *J. Mol. Biol.* **2008**, *379*, 505.
29. Furuta, T. In *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules*; Goeldner, M., Givens, R., Eds.; Wiley-VCH: Weinheim, 2005; p 29.
30. Shembekar, V. R.; Chen, Y.; Carpenter, B. K.; Hess, G. P. *Biochemistry* **2007**, *46*, 5479.
31. Hagen, V.; Frings, S.; Wiesner, B.; Helm, S.; Kaupp, U. B.; Bendig, J. *Chembiochem* **2003**, *4*, 434.
32. Lutz, C.; Otis, T. S.; DeSars, V.; Charpak, S.; DiGregorio, D. A.; Emiliani, V. *Nat. Methods* **2008**, *5*, 821.
33. Olympus Website, 2009.
34. Gandin, V.; Miluzio, A.; Barbieri, A. M.; Beugnet, A.; Kiyokawa, H.; Marchisio, P. C.; Biffo, S. *Nature* **2008**, *455*, 684.
36. Pelletier, J.; Sonenberg, N. *Nature* **1988**, *334*, 320.
37. Jackson, R. J. *Biochem. Soc. Trans.* **2005**, *33*, 1231.
38. Filbin, M. E.; Kieft, J. S. *Curr. Opin. Struct. Biol.* **2009**, *19*, 267.
39. Beaudoin, M. E.; Poirel, V. J.; Krushel, L. A. *Nucleic Acids Res.* **2008**, *36*, 6835.
40. Audigier, S.; Guiramand, J.; Prado-Lourenco, L.; Conte, C.; Gonzalez-Herrera, I. G.; Cohen-Solal, C.; Recasens, M.; Prats, A. C. *RNA* **2008**, *14*, 1852.
41. Lee, V. H.; Healy, T.; Fonseca, B. D.; Hayashi, A.; Proud, C. G. *FEBS J.* **2008**, *275*, 2185.
42. Mamane, Y.; Petroulakis, E.; LeBacquer, O.; Sonenberg, N. *Oncogene* **2006**, *25*, 6416.
43. Jung, M. Y.; Lorenz, L.; Richter, J. D. *Mol. Cell. Biol.* **2006**, *26*, 4277.
44. Macdonald, P. M. *Curr. Biol.* **2004**, *14*, R282.
45. Herbert, T. P.; Fahraeus, R.; Prescott, A.; Lane, D. P.; Proud, C. G. *Curr. Biol.* **2000**, *10*, 793.
46. Fletcher, C. M.; McGuire, A. M.; Gingras, A. C.; Li, H.; Matsuo, H.; Sonenberg, N.; Wagner, G. *Biochemistry* **1998**, *37*, 9.
47. Salaun, P.; Boulben, S.; Mulner-Lorillon, O.; Belle, R.; Sonenberg, N.; Morales, J.; Cormier, P. *J. Cell Sci.* **2005**, *118*, 1385.
48. Marcotrigiano, J.; Gingras, A. C.; Sonenberg, N.; Burley, S. K. *Mol. Cell* **1999**, *3*, 707.
49. Tomoo, K.; Matsushita, Y.; Fujisaki, H.; Abiko, F.; Shen, X.; Taniguchi, T.; Miyagawa, H.; Kitamura, K.; Miura, K.; Ishida, T. *Biochim. Biophys. Acta* **2005**, *1753*, 191.
50. Mahalakshmi, R.; Balaram, P. *Methods Mol. Biol.* **2006**, *340*, 71.
51. Hagen, V.; Dekowski, B.; Kotzur, N.; Lechler, R.; Wiesner, B.; Briand, B.; Beyermann, M. *Chemistry* **2008**, *14*, 1621.
52. Momotake, A.; Lindegger, N.; Niggli, E.; Barsotti, R. J.; Ellis-Davies, G. C. *Nat. Methods* **2006**, *3*, 35.
53. Gingras, A. C.; Raught, B.; Gygi, S. P.; Niedzwiecka, A.; Miron, M.; Burley, S. K.; Polakiewicz, R. D.; Wyslouch-Cieszynska, A.; Aebersold, R.; Sonenberg, N. *Genes Dev.* **2001**, *15*, 2852.
54. Hay, N.; Sonenberg, N. *Genes Dev.* **2004**, *18*, 1926.
55. Kapp, L. D.; Lorsch, J. R. *Annu. Rev. Biochem.* **2004**, *73*, 657.
56. Roux, P. P.; Shahbazian, D.; Vu, H.; Holz, M. K.; Cohen, M. S.; Taunton, J.; Sonenberg, N.; Blenis, J. *J. Biol. Chem.* **2007**, *282*, 14056.
57. Proud, C. G. *Biochem. J.* **2007**, *403*, 217.
58. Hartford, C. M.; Ratain, M. J. *Clin. Pharmacol. Ther.* **2007**, *82*, 381.
59. Choi, J.; Chen, J.; Schreiber, S. L.; Clardy, J. *Science* **1996**, *273*, 239.
60. Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. *J. Mol. Biol.* **1993**, *229*, 105.
61. Guadliana, M. A.; Truesdell, S. U.S.A. Patent, WO 9606847 1996, 21.
62. Gingras, A. C.; Raught, B.; Sonenberg, N. *Prog. Mol. Subcell. Biol.* **2001**, *27*, 143.
63. Raught, B.; Gingras, A. C.; Sonenberg, N. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 7037.
64. Wosnick, J. H.; Shoichet, M. S. *Chem. Mater.* **2008**, *20*, 55.