J|A|C|S COMMUNICATIONS

Published on Web 12/16/2010

Light Regulation of Protein Dimerization and Kinase Activity in Living Cells Using Photocaged Rapamycin and Engineered FKBP

Andrei V. Karginov,[†] Yan Zou,[‡] David Shirvanyants,[§] Pradeep Kota,[§] Nikolay V. Dokholyan,[§] Douglas D. Young,[‡] Klaus M. Hahn,*[,†] and Alexander Deiters*[,‡]

Department of Pharmacology and Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States, and Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States

Received October 26, 2010; E-mail: alex_deiters@ncsu.edu; khahn@med.unc.edu

Abstract: We developed a new system for light-induced protein dimerization in living cells using a photocaged analogue of rapamycin together with an engineered rapamycin binding domain. Using focal adhesion kinase as a target, we demonstrated successful light-mediated regulation of protein interaction and localization in living cells. Modification of this approach enabled light-triggered activation of a protein kinase and initiation of kinase-induced phenotypic changes *in vivo*.

Rapamycin (**Rap**, Figure 1a), also known as sirolimus, is a complex macrolide natural product isolated from the bacterium *Streptomyces hygroscopicus*, found in a soil sample on Easter Island in 1975.[1] Rapamycin mediates heterodimerization of the proteins FKBP12 (FK506 binding protein 12) and FRB (FKBP12 rapamycin binding domain).[2] Due to rapamycin's excellent physiological properties, including good pharmacokinetics, permeability across the blood−brain barrier, and oral bioavailability,[3] it has been used as a small-molecule dimerizer for a wide range of applications in mammalian cells and organisms.[4] Furthermore, the FRB−FKBP12 interaction has proven valuable in a broad range of basic research applications, where it has been engineered to control gene function through rapamycin-induced transcription,[5] protein localization,[5a] protein degradation,[6] and DNA recombination.[7] We recently showed that rapamycin can control kinase activity when an engineered version of FKBP is inserted at a conserved position in the kinase active site.[8] Thus, a photoactivatable analogue of rapamycin represents a unique and important biological research tool, enabling the regulation of heterodimerization and kinase activity using light as a noninvasive regulatory element that can be controlled with high spatial and temporal resolution.

Photoactivatable derivatives of small molecules are typically generated through the installation of a light-removable protecting group, a so-called "caging group", at a site crucial for biological activity of the small molecule.[9] This renders the molecule inactive until the caging group is removed through light irradiation, typically with UV light of 365 nm.[10] The feasibility of this approach has been demonstrated through successful photochemical regulation of numerous small molecules in cellular environments and multicellular organisms.[9,11] Here, we report the synthesis of a photocaged rapamycin analogue (**pRap**, Figure 1a) which, together with an engineered FKBP (iFKBP), enables successful photocontrol of the FKBP−FRB interaction (Figure 1b). The caged rapamycin is applied to regulate both protein dimerization and kinase activity in live cells. Interestingly, caging is not effective with unaltered FKBP but requires the FKBP mutations described here. An analysis of the chemically accessible sites of **Rap** revealed that the methoxy group on C-16 can undergo nucleophilic substitution[13−17] and β-elimination.[12] The hydroxyl groups at C-28 and C-40 can be protected with silyl groups[13,14] and a trifluoromethylsulfonyl group.[15] The lactone at C-34 can be hydrolyzed and eliminated, and, importantly, the hydroxyl group at C-40 can be converted into a carbonate group[15] and be esterified.[14] Thus, C-40 represents the most suitable site for chemical modification with a carbonate-linked caging group that can provide facile installation and quick photolysis. Importantly, based on the crystal structure of the ternary complex of rapamycin, FRB, and FKBP12, the hydroxyl group at C-40 undergoes hydrogen bond formation with the glutamine 53 of FKBP12 (Figure 1c).[16] Thus, we hypothesized that disruption of that hydrogen bond through installation of a sterically demanding α-methyl-6-nitropiperonyloxycarbonyl (MeNPOC) group would prevent protein dimerization.

The caged rapamycin **pRap** was synthesized in one step from **Rap** via chemoselective acylation with the mixed carbonate of α-methyl-6-nitropiperonyl alcohol and *N*-hydroxysuccinimide (MeNPOC-NHS, Figure 1a). The identity of purified **pRap** was confirmed by ^{1}H NMR and HRMS analysis (Supporting Information).

We first tested whether **pRap** could induce dimerization of FKBP12 and FRB. For this we created a GFP-FRB protein fusion and wild-type FKBP12 fused to the N-terminus of focal adhesion kinase (FAK). FAK localizes prominently to focal adhesions in living cells,[17] allowing us to test dimerization *in vivo* by observing rapamycin-mediated translocation of GFP-FRB into focal adhesions. Prior to live cell co-localization studies, the constructs were tested in pull-down assays, comparing the ability of **pRap** and **Rap** to mediate the intracellular dimerization of FKBP-FAK and FRB. Cells expressing both FKBP-FAK and FRB were treated with rapamycin or **pRap** for 1 h, with or without irradiation. Complex formation was assayed by pulling down myc-FKBP-FAK from cell lysates and blotting for GFP-FRB (Supporting Information Figure 1a,b). Surprisingly, both small molecules generated dimerization with similar effectiveness, with or without UV irradiation. These results were further confirmed by an mTOR activity assay (Supporting Information Figure 2). These data showed that the FKBP12−rapamycin−FRB complex was not sufficiently sensitive to rapamycin modification for a successful light-activation approach through photocaging.

We hypothesized that alteration of FKBP12 could be used to render the ternary rapamycin−FKBP−FRB interaction more sensitive to the functional groups of **pRap** affected by photolysis. We tested a recently developed modified FKBP, named iFKBP, that is proposed to have increased structural mobility of the Lys52−Glu54

[†] Department of Pharmacology, University of North Carolina at Chapel Hill.
[‡] Department of Chemistry, North Carolina State University.
[§] Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill.

 10.1021/ja109630v **© 2011 American Chemical Society**

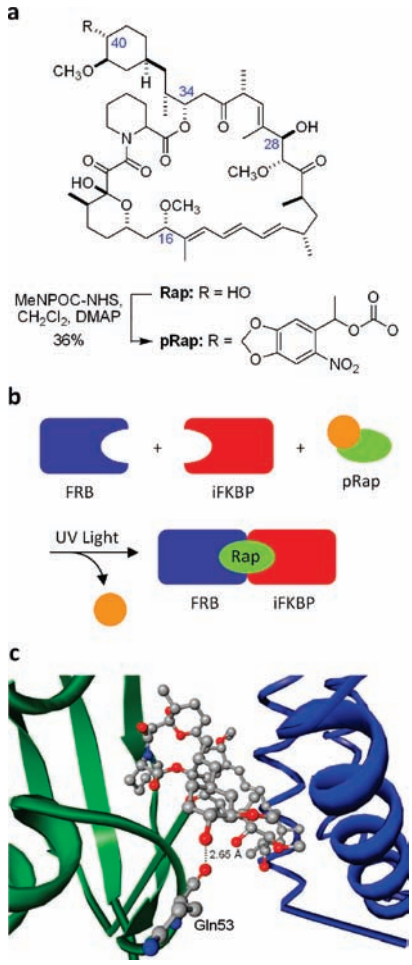

Figure 1. Photocaging of rapamycin. (a) Structure of rapamycin (**Rap**) and its synthetic transformation into caged rapamycin (**pRap**) through selective acylation of the C-40 hydroxyl group with α-methyl-6-nitropiperonyloxycarbonyl *N*-hydroxysuccinimide carbonate (MeNPOC-NHS). (b) Schematic of the light-induced heterodimerization of the proteins FRB and iFKBP using **pRap**. (c) Crystal structure of the ternary complex between rapamycin, FKBP12 (green), and FRB (blue). The 2.65 Å hydrogen bond (possibly mediated through a water molecule) between Gln53 of FKBP12 and the C-40 hydroxyl group of rapamycin is indicated. PDB 2FAP.

loop positioned next to the C-40 hydroxyl group of rapamycin (Figure 1c).[8] Using both an N-terminal iFKBP-FAK fusion (as tested above for FKBP) and a fusion of iFKBP internally, at position 413 of FAK (Figure 2a), we examined whether **pRap** could mediate heterodimerization of iFKBP and FRB in a light-dependent manner. Indeed, **pRap** (at concentrations of up to 20 μM) failed to mediate interaction between iFKBP-FAK and GFP-FRB, while irradiation of **pRap**-treated cells with 365 nm UV light successfully removed the caging group and induced iFKBP-FRB dimerization (Figure 2b−d). Uncaging kinetics were dependent on both light dosage and **pRap** concentration. Importantly, in the presence of **pRap**, translocation of FRB into focal adhesions was observed only upon decaging, indicating successful protein dimerization between FAK-iFKBP and FRB in live cells (Figure 2e,f). These studies demonstrated that **pRap** can effectively mediate light-dependent protein heterodimerization when used with iFKBP rather than FKBP12.

Recently, we developed a new method for the regulation of protein kinases in live cells.[8] Insertion of iFKBP at a structurally conserved position within the catalytic domain of several kinases, including FAK, rendered the kinases inactive. Our previous studies indicate that insertion of the iFKBP increases the mobility of the critical G-loop in the catalytic domain of FAK, resulting

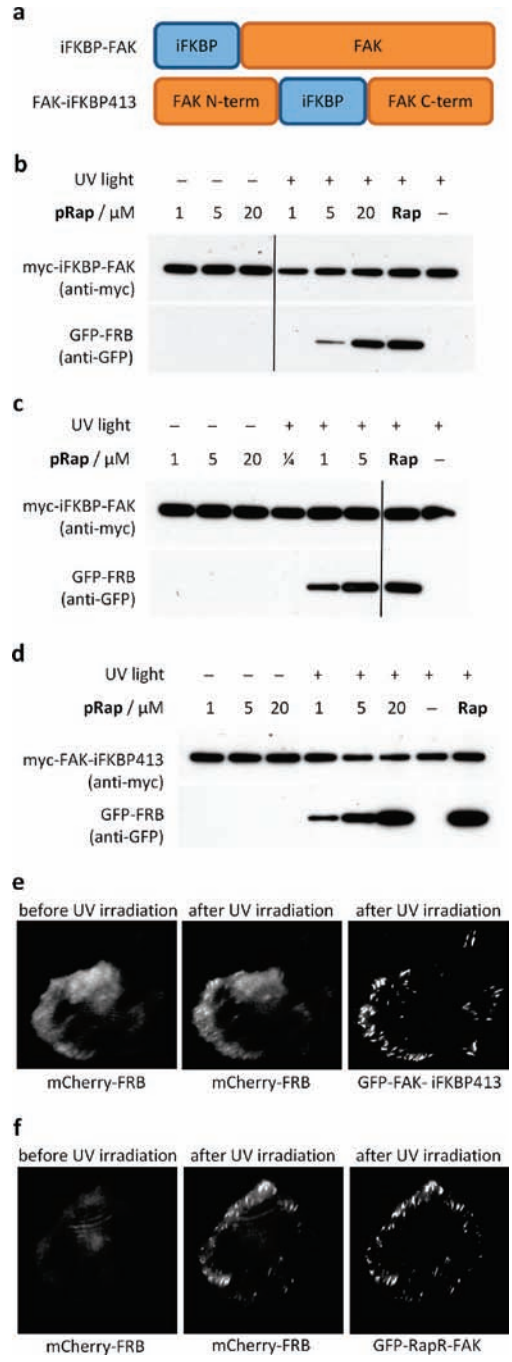

Figure 2. Light-regulated dimerization of iFKBP and FRB. (a) Positions of iFKBP insertions into FAK. (b−d) HEK293T cells co-transfected with GFP-FRB and either myc-iFKBP-FAK (b,c) or myc-FAK-iFKBP413 (d) were treated with either **Rap** (0.5 μM) or the indicated concentrations of **pRap**. Ten minutes after addition of **pRap** or **Rap**, cells were irradiated with 365 nm UV light for 1 min (b,d) or 5 min (c) and incubated for 1 h. Control cells were not irradiated. Myc-iFKBP-FAK was immunoprecipitated from cell lysates using an anti-myc antibody, and co-immunoprecipitation of GFP-FRB was detected by Western Blot using an anti-GFP antibody. (e) HeLa cells co-transfected with GFP-FAK-iFKBP413 and mCherry-FRB were treated with **pRap** (20 μM) for 30 min, followed by UV irradiation (365 nm, 2 min). TIRF images were taken before and after irradiation. (f) HeLa cells co-transfected with GFP-RapR-FAK and mCherry-FRB were treated with **pRap** (5 μM) for 30 min, followed by UV irradiation (365 nm, 2 min). TIRF images were taken before and after irradiation.

in the inhibition of catalytic activity.[8] Formation of an iFKBP−rapamycin−FRB complex through addition of rapamycin significantly restricts iFKBP dynamics, thus stabilizing the

G-loop and rescuing the kinase activity.[8] This was used to achieve specific control of kinases in living cells with high temporal resolution.[8]

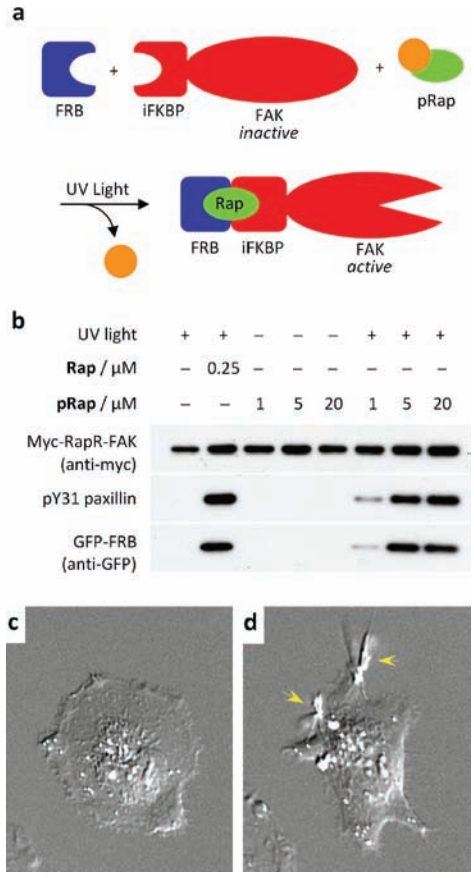

Figure 3. Light-mediated activation of a protein kinase. (a) Schematic of RapR-FAK regulation by **pRap**. (b) Myc-RapR-FAK kinase was co-expressed with GFP-FRB in HEK293T cells. Cells were treated with the indicated amount of **Rap**, caged **pRap**, or DMSO (control). The indicated samples were exposed to UV light (365 nm, 1 min). All cells were incubated at 37 °C for 1 h after treatment. Myc-RapR-FAK was immunoprecipitated using an anti-myc antibody and tested in a kinase assay using an N-terminal fragment of paxillin as a substrate. The level of phosphorylation of paxillin on Tyr31 (probed with anti-phospho-Tyr31 paxillin antibody) indicates the kinase activity. (c,d) HeLa cells co-transfected with GFP-RapR-FAK and Cherry-FRB were treated with **pRap** (1 μM) and imaged before (c) and after (d) UV irradiation (365 nm, 1 min). Arrows indicate formation of large dorsal ruffles stimulated by activated RapR-FAK.

We tested the light-mediated regulation of FAK activity with **pRap** using rapamycin-regulated FAK (RapR-FAK) as a model (Figure 3a). Myc-tagged RapR-FAK was co-expressed with GFP-FRB in HEK293T cells. As in the published validation of RapR-FAK, we examined rapamycin's ability to induce RapR-FAK phosphorylation of the N-terminal fragment of paxillin,[18] a signal transduction adaptor protein and natural FAK substrate. Unlike rapamycin, **pRap** was completely inactive at concentrations of up to 20 μM, producing no detectable paxillin Tyr31 phosphorylation. UV irradiation alone did not activate RapR-FAK in the absence of **pRap**, but irradiation in the presence of **pRap** (1−20 μM) induced activation of RapR-FAK through protein complex formation with FRB (Figure 3b), leading to robust phosphorylation of paxillin. Light-mediated interaction between RapR-FAK and GFP-FRB was further confirmed by co-immunoprecipitation of the two proteins (Figure 3b) and by translocation of FRB to focal adhesions (Figure 2f) upon **pRap** irradiation.

Finally, we examined whether light-induced activation of **pRap** could be used to control cell behavior. FAK activation has been shown to produce large dorsal membrane ruffles.[8] We therefore examined effects of RapR-FAK activation on the membrane dynamics of HeLa cells. In the presence of **pRap**, but without UV irradiation, transfected HeLa cells displayed normal, small peripheral ruffles around the border of the cell (Figure 3c), consistent with inactive RapR-FAK. In contrast, UV irradiation (365 nm) produced very large and dynamic ruffles across the dorsal cell surface (Figure 3d and Supporting Information Movie 1). This UV-induced phenotype was displayed in 40% of analyzed cells (9 of 22 cells), in excellent agreement with effects of regular rapamycin on RapR-FAK (56% positive cells).[8]

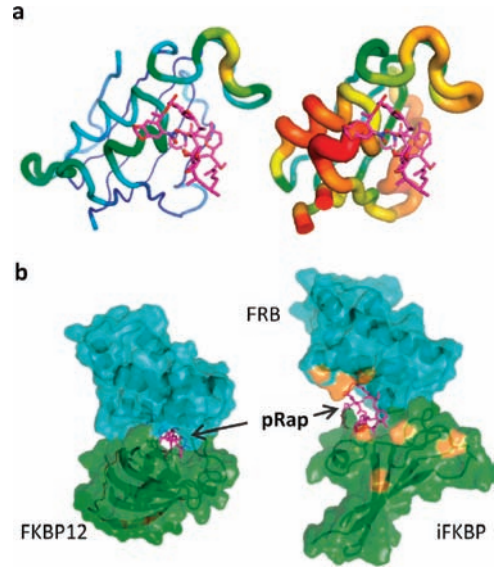

Figure 4. Molecular dynamics simulations of the FKBP12−**pRap**−iFKBP interaction. (a) Tube representations of FKBP12 (left) and iFKBP (right) with **pRap** in the binding pocket. Warmer colors and thicker backbones correspond to fluctuations within the structure. Note the greater mobility of iFKBP. (b) Binding interfaces of the **pRap**, FRB, and FKBP12 or iFKBP complexes. For iFKBP (right) the interface area is reduced due to loss of contacts between the Asp41−Leu49 segment and FRB. In the iFKBP complex, residues that were in contact for the FKBP12 complex are marked in light brown. The segment deleted from FKBP12 to produce iFKBP is marked with dark color in FKBP12.

To explore how the modification of FKBP to iFKBP was able to render **pRap** inactive until it was irradiated with UV light, we performed discrete molecular dynamics simulations.[19] Within the sampled conformations, we identified the dominant ensemble and compared the localization of residues that form the iFKBP-FRB and FKBP12-FRB interfaces. Surprisingly, our simulations demonstrated that binding of the caged rapamycin to iFKBP is at least as strong as that to FKBP12, but the interaction of the added piperonyloxycarbonyl moiety with iFKBP distorts the protein's binding-competent conformations and prevents binding of FRB. The most notable difference between the iFKBP and FKBP complexes is a significant distortion of the FRB-binding interface formed by the segment Asp41−Leu49 (Figure 4a,b and Supporting Information Movies 2−5). The interface area formed between **pRap** and iFKBP is larger than that between **pRap** and FKBP12. Due to its higher structural plasticity, the iFKBP protein is able to deform and create additional contacts with **pRap**, which is unachievable by the more rigid and stable FKBP12. On the basis of these simulations, we suggest that strong binding between iFKBP and **pRap** distorts the FRB-binding interface in FKBP12 and thus prevents further binding to FRB.

In summary, we have developed a new photocaged analogue of rapamycin, **pRap**, which can be used together with iFKBP, an engineered version of FKBP12, for the light-mediated regulation of protein dimerization. We demonstrated applications of this new dimerization system by modulating protein interactions for two different FAK-iFKBP fusions in living cells. Furthermore, we achieved light-mediated activation of an engineered protein kinase, FAK, and demonstrated light-induced changes in cell behavior characteristic of this kinase. Rapamycin-mediated protein dimerization and regulation of kinases have lacked the precise spatial and temporal control of light-mediated processes. Our new caged rapamycin approach will significantly enhance the many existing methodologies that use rapamycin for the regulation of protein activity in cells and multicellular organisms. We are currently exploring the synthesis of caged, orthogonal rapalogues and two-photon-activated caged rapamycin.

Acknowledgment. This research was supported by the NIH (A.D., GM079114; K.M.H., cell migration consortium grant GM64346; N.V.D., GM080742 and the ARRA supplements GM080742-03S1 and GM066940-06S1).

Supporting Information Available: Synthetic protocols and proton NMR spectrum of caged rapamycin, protocols for cell culture experiments, supporting movies, detailed information on the molecular dynamics simulations, and complete ref 15. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Vezina, C.; Kudelski, A.; Sehgal, S. N. *J. Antibiot.* **1975**, *28*, 721. (b) Sehgal, S. N.; Baker, H.; Vezina, C., *J. Antibiot.* **1975**, *28*, 727. (c) Baker, H.; Sidorowicz, A.; Sehgal, S. N. Vezina, C. *J. Antibiot.* **1978**, *31*, 539. (d) Singh, K.; Sun, S.; Vezina, C. *J. Antibiot.* **1979**, *32*, 630.
(2) Huang, S.; Bjornsti, M. A.; Houghton, P. J. *Cancer Biol. Ther.* **2003**, *2*, 222.
(3) Abraham, R. T.; Wiederrecht, G. J. *Annu. Rev. Immunol.* **1996**, *14*, 483.
(4) Pollock, R.; Clackson, T. *Curr. Opin. Biotechnol.* **2002**, *13*, 459.
(5) (a) Bayle, J. H.; Grimley, J. S.; Stankunas, K.; Gestwicki, J. E.; Wandless, T. J.; Crabtree, G. R. *Chem. Biol.* **2006**, *13*, 99. (b) Pollock, R.; Issner, R.; Zoller, K.; Natesan, S.; Rivera, V. M.; Clackson, T. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13221.
(6) (a) Edwards, S. R.; Wandless, T. J. *J. Biol. Chem.* **2007**, *282*, 13395. (b) Maynard-Smith, L. A.; Chen, L. C.; Banaszynski, L. A.; Ooi, A. G.; Wandless, T. J. *J. Biol. Chem.* **2007**, *282*, 24866.
(7) Keith, C. T.; Schreiber, S. L. *Science* **1995**, *270*, 50.
(8) Karginov, A. V.; Ding, F.; Kota, P.; Dokholyan, N. V.; Hahn, K. M. *Nat. Biotechnol.* **2010**, *28*, 743.
(9) (a) Riggsbee, C. W.; Deiters, A. *Trends Biotechnol.* **2010**, *28*, 468. (b) Mayer, G.; Heckel, A. *Angew. Chem., Int. Ed.* **2006**, *45*, 4900. (c) Young, D. D.; Deiters, A. *Org. Biomol. Chem.* **2007**, *5*, 999. (d) Deiters, A. *Chembiochem* **2010**, *11*, 47. (e) Deiters, A. *Curr. Opin. Chem. Biol.* **2009**, *13*, 678. (f) Tang, X.; Dmochowski, I. J. *Mol. Biosyst.* **2007**, *3*, 100. (g) Lawrence, D. S. *Curr. Opin. Chem. Biol.* **2005**, *9*, 570. (h) Curley, K.; Lawrence, D. S. *Curr. Opin. Chem. Biol.* **1999**, *3*, 84. (i) Lee, H. M.; Larson, D. R.; Lawrence, D. S. *ACS Chem. Biol.* **2009**, *4*, 409. (j) Dorman, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64. (k) Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755.
(10) (a) Dong, Q.; Svoboda, K.; Tiersch, T. R.; Monroe, W. T. *J. Photochem. Photobiol. B* **2007**, *88*, 137. (b) Schindl, A.; Klosner, G.; Honigsmann, H.; Jori, G.; Calzavara-Pinton, P. C.; Trautinger, F. *J. Photochem. Photobiol. B* **1998**, *44*, 97. (c) Robert, C.; Muel, B.; Benoit, A.; Dubertret, L.; Sarasin, A.; Stary, A. *J. Invest. Dermatol.* **1996**, *106*, 721.
(11) (a) 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. (b) Cambridge, S. B.; Geissler, D.; Calegari, F.; Anastassiadis, K.; Hasan, M. T.; Stewart, A. F.; Huttner, W. B.; Hagen, V.; Bonhoeffer, T. *Nat. Methods* **2009**, *6*, 527. (c) Sauers, D. J.; Temburni, M. K.; Biggins, J. B.; Ceo, L. M.; Galileo, D. S.; Koh, J. T. *ACS Chem. Biol.* **2010**, *5*, 313. (d) Young, D. D.; Garner, R. A.; Yoder, J. A.; Deiters, A. *Chem. Commun.* **2009**, *568.* (e) Young, D. D.; Deiters, A. *Angew. Chem., Int. Ed.* **2007**, *46*, 4290. (f) Link, K. H.; Cruz, F. G.; Ye, H. F.; O'Reilly, K, E.; Dowdell, S.; Koh, J. T. *Bioorg. Med. Chem.* **2004**, *12*, 5949. (g) Cruz, F. G.; Koh, J. T.; Link, K. H. *J. Am. Chem. Soc.* **2000**, *122*, 8777. Over the course of our studies, a C16 caged rapamycin has been reported: (h) Sadovski, O.; Jaikaran, A. S.; Samanta, S.; Fabian, M. R.; Dowling, R. J.; Sonenberg, N.; Woolley, G. A. *Bioorg. Med. Chem.* **2010**, *18*, 7746. Interestingly, a previously synthesized C16 nitrobenzyl caged rapamycin was fully active in our assays.
(12) Grinfeld, A. A.; Caufield, C. E.; Schiksnis, R. A.; Mattes, J. F.; Chan, K. W. *Tetrahedron Lett.* **1994**, *35*, 6835.
(13) Nelson, F. C.; Stachel, S. J.; Mattes, J. F. *Tetrahedron Lett.* **1994**, *35*, 7557.
(14) Ayral-Kaloustian, S.; Gu, J. X.; Lucas, J.; Cinque, M.; Gaydos, C.; Zask, A.; Chaudhary, I.; Wang, J. Y.; Di, L.; Young, M.; Ruppen, M.; Mansour, T. S.; Gibbons, J. J.; Yu, K. *J. Med. Chem.* **2010**, *53*, 452.
(15) Wagner, R.; et al. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5340.
(16) (a) Choi, J.; Chen, J.; Schreiber, S. L.; Clardy, J. *Science* **1996**, *273*, 239. (b) Liang, J.; Choi, J.; Clardy, J. *Acta Crystallogr. D: Biol. Crystallogr.* **1999**, *55*, 736.
(17) Schaller, M. D.; Borgman, C. A.; Cobb, B. S.; Vines, R. R.; Reynolds, A. B.; Parsons, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5192.
(18) (a) Deakin, N. O.; Turner, C. E. *J. Cell Sci.* **2008**, *121*, 2435. (b) Schaller, M. D. *J. Cell Biol.* **2004**, *166*, 157.
(19) (a) Dokholyan, N. V.; Buldyrev, S. V.; Stanley, H. E.; Shakhnovich, E. I. *Fold. Des.* **1998**, *3*, 577. (b) Ding, F.; Tsao, D.; Nie, H.; Dokholyan, N. V. *Structure* **2008**, *16*, 1010.

JA109630V