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Optochemical Activation of Kinase Function in Live Cells

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Summary

Manipulation of protein kinase activity is widely used to dissect signaling pathways controlling physiological and pathological processes. Common methods often cannot provide the desired spatial and temporal resolution in control of kinase activity. Regulation of kinase activity by photocaged kinase inhibitors has been successfully used to achieve tight temporal and local control, but inhibitors are limited to inactivation of kinases, and often do not provide the desired specificity. Here we report detailed methods for light-mediated activation of kinases in living cells using engineered rapamycin-regulated kinases (RapR-kinases) in conjunction with a photocaged analog of rapamycin.

Keywords

Kinase; Phosphorylation; Rapamycin; FKBP12; Caging; Light Activation

1. Introduction

Protein kinases play a central role in the regulation of signaling networks critical for cell function. The most common methods for the regulation of kinases include pharmacological inhibitors, overexpression of mutants, and downregulation of protein expression by genetic manipulation or siRNA. These methods are valuable, but suffer from numerous limitations. Pharmacological inhibitors are available only for a limited number of kinases and often cannot provide the desired specificity. It is especially difficult to achieve high inhibitor selectivity for a kinase that has several homologs or isoforms. Downregulation or overexpression of kinases provides essentially no temporal resolution, and affects function of the whole protein rather than “surgically” regulating catalytic activity alone. The time required for genetic modification to result in altered protein allows the cell to compensate for (and adapt to) the perturbation. More recently, a chemical-genetic approach has overcome some of these limitations by using specifically designed inhibitors for kinases with a drug-sensitizing mutation (1). However, this method requires separate inhibition or downregulation of the endogenous, wild-type form of the modified kinase, involving elaborate optimization and potentially affecting activity of other endogenous kinases.

Importantly, none of these methods enable specific activation of kinases with controlled timing. Several methods that do enable activation are limited to a small subset of kinases that either can be activated by dimerization (2) or can be modified for regulation by high concentrations of imidazole (5 mM) (3). We have recently developed a new broadly applicable method for engineered allosteric regulation of protein kinases in live cells (4–6). Insertion of an engineered allosteric switch, the iFKBP domain, at a structurally conserved position within the catalytic domain renders the modified kinase inactive. Treatment with rapamycin or its non-immunosuppressive analogs (iRap, AP21967; Clontech) triggers interaction with a small FKBP-rapamycin-binding domain (FRB) and restores the activity of the kinase (Fig. 1A). The reagents used in this method are either genetically encoded or membrane permeable, enabling ready application in a wide range of systems. Based on the structural similarity of catalytic domains among most known protein kinases, this method will likely be applicable to a wide variety of kinases. We have already developed rapamycin-regulated (RapR) analogs of different kinases, representing both Tyr and Ser/Thr families, namely FAK, Src, and p38.

To achieve light-mediated control of RapR-kinases we generated a photo-activatable analog of rapamycin (pRap) (7). Photo-activatable derivatives of small molecules are typically generated through conjugation of a light-removable protecting group, a so called “caging group”, at a site crucial for biological activity of the small molecule (8–18). Ideally, this renders the molecule completely inactive and unable to induce iFKBP/FRB dimerization, until the caging group is removed through light-irradiation, typically with non-toxic (19–21) UV light of 365–405 nm. We constructed the photocaged rapamycin analog (pRap) by protecting the hydroxyl group at the C-40 position of rapamycin with a sterically demanding, light-removable nitropiperonyloxycarbonyl (NPOC) group (Fig. 1B). Treatment with pRap up to 20 μ M does not trigger interaction with FRB and activation of RapR-kinase. However, irradiation with 360nm light leads to rapid pRap uncaging and robust activation of RapR-kinase upon interaction with FRB (Fig. 1C) (7). The experimental details of applying this methodology to the optochemical activation of focal adhesion kinase (FAK) are described here. FAK localizes prominently to focal adhesions in living cells and we use changes in cell behavior characteristic of this kinase to demonstrate efficient light activation. Similar strategies can also be applied for activation of other RapR-kinases. In case a different RapR-kinase is used, appropriate modifications to the protocol should be made to test the activity of the kinase. Beyond the activation of kinase function, pRap should be applicable to induce iFKBP/FRB-mediated protein dimerization and thus should provide a general light-switch to control of various cellular processes.

2. Materials

Prepare all buffers and solutions with ultrapure water. Store all buffers at 4°C unless specified otherwise.

2.1. pRap synthesis

1. Chemicals were obtained from commercial sources (e.g., Sigmal-Aldrich, Fisher, VWR): *N,N'*-disuccinimidyl carbonate, dimethylaminopyridine (DMAP),

rapamycin, triethylamine (TEA), acetonitrile (CH₃CN), dichloromethane (CH₂Cl₂), ethyl acetate, and hexanes.

2. Solvents were distilled and stored over molecular sieves (4 Å) prior to use. Triethylamine (TEA), diisopropylethylamine (DIPEA), and CH₃CN were distilled from calcium hydride. Dichloromethane (CH₂Cl₂) was dried by a MB SPS Compact solvent purification system.

2.2. *In vitro* kinase assay

1. DNA constructs: pEGFP-FRB, pmyc-RapR-FAK plasmids (available at Addgene) dissolved in water or TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) at concentrations no less than 200 µg/ml. Store at –20 °C.
2. Cell culture medium: Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L glucose, 4 mM L-glutamine, and 110 mg/L sodium pyruvate) cell culture medium containing 10% (v/v) Fetal Bovine Serum (FBS) (*see* Note 1). Store at 4°C.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH of 7.4. Weigh 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄. Dissolve in 800 ml of water. Adjust pH to 7.4 using HCl. Adjust total volume to 1 L with water.
4. FuGene6 transfection reagent (Promega) (*see* Note 2).
5. 6-well tissue culture plates.
6. Cell scraper.
7. Immunoprecipitation: ProteinG-coupled agarose beads (Millipore or other manufacturer), 50% slurry. Store at 4 °C.
8. Antibodies: JL8 anti-GFP antibody (Clontech), Anti-phospho-Tyr31 paxillin antibody (Invitrogen), 4A6 anti-myc antibody (1 mg/ml, Millipore), Peroxidase-conjugated secondary antibody. Follow storage conditions recommended by manufacturer.
9. Reagents and equipment required for SDS-polyacrylamide gel electrophoresis of proteins.
10. Reagents and equipment required for protein transfer onto PVDF membrane.
11. Lysis Buffer: 20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 1 mM EGTA, 1% NP40 (IGEPAL), 1 mM NaF, 0.2 mM Na₃VO₄. Keep on ice (*see* Note 3).

¹Cell media conditions are determined by the specific cell line used in the experiment. The medium described here is recommended for the HEK293 and HeLa cells used in our experiments.

²Other transfection reagents can be used. If a different transfection protocol is used, it is recommended to test transfection efficiency before setting up the described experiments. If transfection efficiency is lower than 30%, either larger quantities of cells should be used or an alternative transfection protocol should be tested.

³Buffer containing 20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 1 mM EGTA, and 1% NP40 should be prepared separately and stored at 4 °C. Stock solutions of 0.5 M NaF and 0.2 M Na₃VO₄ in water should be stored at –20°C. Complete Lysis Buffer should be prepared on the day of the experiment and should be stored on ice.

12. Wash Buffer: 20 mM Hepes-KOH, 50 mM KCl, 100 mM NaCl, 1 mM EGTA, 1% NP40 (IGEPAL, Sigma-Aldrich), pH 7.8. Keep on ice.
13. Kinase Reaction Buffer: 25 mM HEPES pH7.5, 5 mM MgCl₂, 5 mM MnCl₂, 0.5 mM EGTA, 0.005% BRIJ-35 (nonionic detergent, Fisher BioReagents) (*see* Note 4). Keep on ice.
14. Paxillin/ATP mix: 0.1 mM ATP and 0.05 mg/ml purified GST-paxillin N-terminal fragment in Kinase Buffer (*see* Note 5). A protocol for purification of the GST-tagged N-terminus of paxillin has been described elsewhere (22).
15. Rapamycin and pRap: dissolved in DMSO at 0.25 mM for rapamycin, 1 mM, 5 mM and 20 mM stocks for pRap. Store at −20 °C.
16. UV irradiation: UVP LMW-20 transilluminator (8W) (*see* Note 6).
17. HEK293 cells (ATCC).
18. 1 mg/ml Bovine Serum Albumin (BSA) dissolved in Lysis Buffer (without NaF and Na₃VO₄). Store at −20 °C.

2.3. Components for live cell imaging

1. HeLa cells (ATCC).
2. DNA constructs: pGFP-RapR-FAK and pmCherry-FRB plasmids (Addgene), dissolved in water or TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at concentration no less than 200 µg/ml. Store at −20 °C.
3. FuGene6 transfection reagent (Promega) (*see* Note 1).
4. Cell culture medium: Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L glucose, 4 mM L-glutamine, and 110 mg/L sodium pyruvate) containing 10% (v/v) Fetal Bovine Serum (FBS).
5. Live cell imaging medium: L15 Leibovitz Medium (Invitrogen) supplemented with 5% FBS (*see* Note 7).
6. Mineral oil, sterile filtered, suitable for mouse embryo cell culture (Sigma-Aldrich).
7. 25 mm round glass coverslips, 0.13–0.17 mm thick (Fisher Scientific). Store in 70% ethanol solution.
8. 1 mg/ml Fibronectin stock solution dissolved in 0.5 M NaCl, 0.05 M Tris, pH 7.5 (*see* Note 8).

⁴Buffer containing 25 mM HEPES pH 7.5, 5 mM MgCl₂, 0.5 mM EGTA, and 0.005% BRIJ-35 should be prepared separately and stored at 4°C. Stock solutions of MnCl₂ (1 M) in water should be stored at −20 °C. If precipitation is observed in MnCl₂ solution, then new stock solutions should be prepared. Complete Kinase Reaction Buffer should be prepared on the day of the experiment and stored on ice.

⁵Paxillin-ATP mix should be prepared right before the experiment by mixing 100 mM ATP stock solution and stock solution of the purified GST-paxillin N-terminal fragment in Kinase Buffer. The mixture should be kept on ice.

⁶An equivalent UV transilluminator can be used for this experiment, but uncaging efficiency should be determined by irradiating cells for different periods of time.

⁷L15 medium (4 °C) and FBS should be stored separately (−20 °C). On the day of the experiment, prepare a fresh mix of L15 medium and FBS. A minimum of 1 ml will be needed per experiment.

⁸Fibronectin solution in PBS should be prepared freshly at the time of application.

9. Attofluor® cell chamber (Invitrogen) (*see* Note 9).
10. 35 mm tissue culture plates.
11. Microscope equipped with an objective-based total internal reflection fluorescence (TIRF) system, a 60× TIRF objective, CCD camera, a high pressure mercury arc light source, laser lines with excitation at 488 nm and 590 nm for TIRF imaging, and an open heated chamber (*see* Note 10).
12. 5 mM pRap dissolved in DMSO. Store at −20 °C.
13. UV irradiation: UVP UVGL-25 hand-held UV lamp (4 W) (*see* Note 6).

3. Methods

All centrifugation steps can be performed in a benchtop centrifuge at room temperature unless indicated otherwise.

3.1. pRap Synthesis

3.1.1. Synthesis of α -methyl-6-nitropiperonyl succinimidyl carbonate (MeNPOC-NHS)

1. 1-(3,4-(Methylenedioxy-6-nitrophenyl)ethanol was synthesized as reported (23) and 200 mg (0.947 mmol) were dissolved in 5 ml of dry CH₃CN (*see* Note 23).
2. To the solution were added *N,N'*-disuccinimidyl carbonate (485 mg, 1.894 mmol) (*see* Note 24) and TEA (0.396 ml, 2.840 mmol).
3. The reaction was stirred at room temperature overnight.
4. The solvent was removed under reduced pressure and the product was directly purified by column chromatography on silica gel (eluted with hexanes/ethyl acetate 5:1), delivering 296 mg (89% yield) of MeNPOC-NHS as a light yellow solid.
5. Analytical data: ¹H NMR (300 MHz, CDCl₃) δ 7.49 (s, 1H), 7.09 (s, 1H), 6.39 (q, *J* = 6.4 Hz, 1H), 6.14–6.12 (m, 2H), 2.79 (s, 4H), 1.73 (d, *J* = 6.4 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 168.7, 153.0, 150.8, 148.0, 141.6, 133.2, 105.9, 105.6, 103.5, 76.5, 25.6, 22.3 (*see* Note 25); high-resolution mass spec calculated C₁₄H₁₂N₂NaO₉ 375.04405, found 375.0430.

3.1.2. Synthesis of MeNPOC-caged rapamycin (pRap)

1. Under argon, rapamycin (20.0 mg, 0.022 mmol) was dissolved in dry DCM (0.6 ml) (*see* Note 23).

⁹Other inverted epifluorescence microscopes suitable for live cell imaging can be used. The instrument should allow for addition of reagents during cell imaging.

¹⁰We routinely use an Olympus IX-81 microscope equipped with an objective-based total internal reflection fluorescence (TIRF) system and a PlanApo N 60× TIRFM objective (NA 1.45). All images are collected using a Photometrix CoolSnap ES2 CCD camera controlled by Metamorph software. The 488 nm line from an omnichrome series 43 Ar/Kr laser and the 594 nm line from a Cobolt Mambo continuous-wave diode-pumped solid-state laser are used for TIRF imaging. Illumination for epifluorescence images was provided from a high pressure mercury arc light source.

²³All reactions were performed in flame-dried glassware under a nitrogen atmosphere and stirred magnetically unless indicated.

²⁴All commercially available chemicals and reagents were used without further purification unless indicated.

²⁵NMR spectra were recorded using Varian Mercury (300 MHz and 400 MHz) instruments.

2. DMAP (5.4 mg, 0.044 mmol) (*see* Note 24) and MeNPOC-NHS (39.0 mg, 0.109 mmol) were added.
3. The reaction mixture was stirred at room temperature for 24 h.
4. The volatiles were evaporated under reduced pressure, and the product was purified by column chromatography on silica gel (eluted with DCM/ethyl acetate 10:1, 5:1, 2:1, 1:1), delivering 9.1 mg (36% yield) of pRap as a light yellow solid. No attempts were made to separate the two generated diastereomers.
5. Analytical data: ¹H NMR (400 MHz, CDCl₃) δ 7.48 (s, 1H), 7.05 (s, 1H), 6.44-6.03 (m, 7H), 5.94-5.83 (m, 1H), 5.60-5.47 (m, 1H), 5.43 (m, 1H), 5.38-5.31 (m, 1H), 5.20-5.08 (m, 1H), 4.50-4.38 (m, 1H), 4.14-4.09 (m, 2H), 3.98-3.73 (m, 2H), 3.67-3.55 (m, 2H), 3.35-3.31 (m, 7H), 3.13-3.03 (m, 5H), 2.87-2.50 (m, 3H), 2.40-2.11 (m, 2H), 2.03-1.52 (m, 15H), 1.47-1.32 (m, 6H), 1.24-0.86 (m, 24H) (*see* Note 25); high-resolution mass spec calculated for [M + Na]⁺ C₆₁H₈₆N₂NaO₁₉ 1173.5723, found 1173.5721.

3.2. Light-mediated activation of RapR-FAK kinase. Assessment of activity using an *in vitro* kinase assay

1. Distribute 10⁶ HEK293 cells per well into two 6-well plates (5 wells in one plate and 3 wells in another) in 2 ml DMEM media with 10% FBS and grow in a 37°C, 5% CO₂ incubator overnight. Cells should be 60–80% confluent for optimal transfection.
2. Using 1:1 ratio co-transfect HEK293T cells with pEGFP-FRB and myc-RapR-FAK (all wells). Perform transfection using FuGene6 reagent according to the manufacturers' recommendations (2 µg of DNA/6 µl FuGene6 per well). Other equivalent transfection methods can also be used (*see* Note 2). Incubate in a 37°C, 5% CO₂ incubator overnight.
3. On the day of the experiment, prepare ProteinG-coupled agarose beads for incubation with the 4A6 anti-myc antibody. Transfer 80 µl of bead suspension into a fresh 1.5 ml tube (10 µl of bead suspension is sufficient for each immunoprecipitation (IP) sample, 8 IP samples in this experiment) (*see* Note 11).
4. Wash beads with 1 ml of Lysis Buffer (*see* Note 12). Resuspend beads in 400 µl of Lysis Buffer containing 1 mg/ml BSA and add 4 µl of 4A6 antibody (use 0.5 µl of antibody per IP).
5. Incubate beads at 4 °C for 1–2 hours. Wash beads two times with 1 ml of Lysis Buffer (*see* Note 12) and resuspend in 400 µl of Lysis Buffer (50 µl of Lysis Buffer for each IP). Aliquot 50 µl of beads into fresh 1.5 ml tubes for incubation with cell lysates.

¹¹Agarose beads need to be well resuspended before removing an aliquot. The tip of the pipetman tip can be cut in order to prevent it from being clogged by agarose clumps.

¹²To wash the agarose beads, add the buffer to the tube, resuspend by vortexing, centrifuge at 1500 g for 1 min and remove the supernatant.

6. Replace media on the transfected cells with 2 ml of fresh DMEM media containing 10% FBS. For both plates treat cells with three different concentrations of pRap: 1 μ M, 5 μ M, and 20 μ M (add 2 μ L of the corresponding stock solution of pRap, one well for each plate, mix by gentle agitation) (*see* Note 13).
7. Treat one well of transfected cells with 250 nM rapamycin (2 μ L of 0.25 μ M stock, positive control) and another well with 2 μ L of DMSO (solvent control). Both wells should be in the 6-well dish with 5 wells of plated and transfected cells. Incubate all cells in a 37°C, 5% CO₂ incubator for 10 minutes.
8. Irradiate plate with 5 wells of cells with 365 nm light by placing it on a UVP LMW-20 transilluminator for 1 min (*see* Note 14). Keep other plate in the incubator without irradiation; this will be your negative control to be compared to RapR-FAK induced by uncaged pRap.
9. Incubate all cells in a 37°C, 5% CO₂ incubator for 1 hour.
10. Wash all cells with cold PBS on ice (~3–4 ml of PBS per well) (*see* Note 15). Aspirate as much PBS as possible after the wash.
11. Add 300 μ L of Lysis Buffer to each well. Scrape cells from the plate, collect cell lysates into 1.5 ml tubes and spin 10 min at 1000 g and 4°C.
12. Collect 20 μ L of supernatant for protein gel electrophoresis analysis (*see* Note 16). Transfer the remaining supernatant into the tubes containing 50 μ L of beads prepared in the previous step.
13. Incubate lysates with the beads at 4°C for 1.5–2 hours with constant agitation.
14. Wash beads two times with 0.5 ml of Wash Buffer and two times with 0.5 ml of Kinase Buffer (*see* Note 12). Remove all buffer from beads after last wash.
15. Add 40 μ L of Kinase buffer per tube. Resuspend the beads and transfer 20 μ L of each sample into a fresh 1.5 ml tube for the kinase reaction.
16. Add 10 μ L of Paxillin/ATP mix and incubate 10 min at 37 °C shaking (*see* Note 17).
17. Stop the reaction by the addition of 40 μ L 2 $\times$ Laemmli protein sample buffer. Incubate at 95–100 °C for 5 min. Cool samples to room temperature. Run on a protein SDS-polyacrylamide gel.
18. Perform western blot analysis using a 4A6 anti-myc antibody for detection of myc-FAK variants, anti-phospho-Tyr31 paxillin antibody for assessment of substrate phosphorylation, and anti-GFP JL8 antibody for detection of GFP-FRB (Fig. 2).

¹³Adding reagents and mixing media needs to be done very carefully, as HEK293 cells may detach if agitated too vigorously.

¹⁴Protect your eyes and skin from irradiation with UV light by wearing appropriate protective goggles, gloves, and lab coat.

¹⁵Add PBS slowly to the side of the well to avoid cell detachment.

¹⁶Run lysate samples on a separate gel. Transfer onto PVDF membrane and probe with anti-myc and anti-GFP antibody to check expression of myc-RapR-FAK and GFP-FRB.

¹⁷We recommend to add paxillin/ATP mix to the side of each reaction tube, then spin all tubes briefly at 4000 rpm to add the mix to the beads in all reactions simultaneously and incubate reactions in a thermomixer set to 37 °C.

3.2. Light-mediated activation of RapR-FAK kinase – Live cell imaging

1. Plate 200,000 HeLa cells in a 35 mm tissue culture dish and grow overnight in a 37 °C, 5% CO₂ incubator. Cell confluency should be 50–70% the next morning.
2. Co-transfect HeLa cells with 0.5 µg of GFP-FRB plasmid and 1.5 µg of pEGFP-RapR-FAK plasmid using 4 µl of FuGene6 according to the manufacturers' recommendations (*see* Note 2). Incubate overnight at 37 °C, 5% CO₂.
3. Place a glass coverslip in 35 mm tissue culture plates or 6-well plates. Wash with 2–4 ml of PBS. Incubate the coverslip in 2 ml of 5 mg/ml fibronectin solution in PBS at 37 °C overnight. Wash the coverslip with PBS and add 2 ml of DMEM media with 10% FBS.
4. Plate transfected HeLa cells onto fibronectin-coated coverslip. Incubate in DMEM/10% FBS medium for 2 hours at 37 °C, 5% CO₂ (*see* Note 18).
5. Preincubate mineral oil and L15 media supplemented with 5% FBS in a tissue culture incubator (37 °C, 5% CO₂) for at least 1 hour before imaging.
6. Wash the coverslip with PBS and place it in an Attotfluor® cell chamber. Add 0.9 ml of L15 Leibovitz Media with 5% FBS and cover it with 1 ml of mineral oil (*see* Note 19). Place cell chamber onto heated stage of the microscope and select cells co-expressing GFP-RapR-FAK and mCherry-FRB (*see* Note 20).
7. Image cells co-expressing GFP-RapR-FAK and mCherry-FAK, taking images every minute for 120 minutes. Mix 1 µL of 5 µM pRap solution with 100 µL of L15 Leibovitz Media. Add pRap solution to the cells (final concentration of 5 µM) 30 min after imaging has begun (*see* Note 21). Decage pRap 30 min after its addition by placing a UVP UVGL-25 hand-held UV lamp 2–3 cm above the cell chamber and irradiating with UV light for 1 min (*see* Note 14). Continue imaging for the remaining 60 min. DIC imaging can be used to monitor cell movement and overall changes in cell morphology (i.e., protrusion formation and cell shape) (Fig. 3A). Epifluorescence can be used to monitor RapR-FAK, FRB or any other fluorescently labeled co-transfected protein. TIRF imaging will reveal translocation of Cherry-FRB to the focal adhesion sites demonstrating interaction between Cherry-FRB and GFP-RapR-FAK induced by uncaging of pRap (Fig. 3B) (*see* Note 22).

¹⁸It takes 1–2 hours for HeLa cells to attach to the coverslips and spread.

¹⁹Addition of oil on top of the media prevents evaporation, but still allows for addition of reagents.

²⁰A microscope equipped with a motorized stage enables consecutive imaging of several cells by selecting and logging the positions of cells expressing GFP-RapR-FAK and mCherry-FRB. The number of positions depends on the time to take all the images at one position and move to the next one.

²¹Mix pRap with the media right before adding it to the cells. Make sure you penetrate the oil layer when adding pRap to the cells. Imaging the first 30 min without pRap is required to establish a baseline in order to determine if addition of pRap causes any changes in cell behavior or changes in protein localization.

²²FAK localizes to focal adhesions at the side of the cell where it attaches to the dish. In the absence of active rapamycin, FRB will be diffusely localized throughout the cell.

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Fig. 1. Engineered control of RapR-kinases. (A) Schematic of rapamycin-mediated control of kinases. (B) Structure of rapamycin (Rap) and synthesis of photocaged rapamycin (pRap) through selective acylation of the C-40 hydroxyl group with methyl-nitro-piperonyloxycarbonyl *N*-hydroxysuccinimide carbonate (MeNPOC-NHS). (C) Schematic of the light-induced activation of RapR-kinases using pRap.

Fig. 2.

Light-induced activation of RapR-FAK. Representative results of an *in vitro* kinase assay.

The level of phosphorylation of paxillin on Tyr31 (probed with an anti-phospho-Tyr31 paxillin antibody) indicates kinase activity.

Fig. 3.

Effect of light-induced activation of RapR-FAK in live cells and its dimerisation with FRB. (A) DIC images of HeLa cells expressing GFP-RapR-FAK and mCherry-FRB before and after uncaging of pRap. Arrows indicate formation of large dorsal ruffles stimulated by activated RapR-FAK. (B) Localization of mCherry-FRB before and after uncaging of pRap. Images were taken using TIRF microscopy.