Antibodies and reagents.

Anti-phospho-paxillin (Tyr31), anti-phospho-FAK (Tyr397), anti-phospho-Src (Tyr418) and anti-GFP antibodies were purchased from Invitrogen. Anti-myc antibodies and IgG-coupled agarose beads were purchased from Millipore. Anti-paxillin antibodies were a gift from M. Schaller. Rapamycin was purchased from Sigma. All restriction enzymes were purchased from New England Biolabs. iRap was a gift from T. Wandless. AP21697 was provided by Ariad Pharmaceuticals.

Molecular biology.

The construct for myc-tagged mouse FAK was a gift from S. K. Hanks. The construct expressing the GST-tagged N-terminal fragment of paxillin was a gift from M. Schaller. The mouse Src and was purchased from Upstate. The flag-tagged mouse p38α, human FKBP12 and FRB domain of human FRAP1 DNA constructs were a gift from G. Johnson. The iFKBP domain consisted of amino acids Thr22 through Glu108 of human FKBP12. Insertion of wild-type FKBP12 or iFKBP at the ends or in the middle of FAK, p38 and Src genes was performed using a modification of the QuikChange site-directed mutagenesis kit (Stratagene). The FKBP12 and iFKBP inserts were created by PCR such that their 5′- and 3′-end sequences annealed at the desired insertion site within the p38, Src and FAK genes. The PCR products were used as mega-primers for QuikChange mutagenesis reactions. In the RapR Src construct, the iFKBP insert contained GPG linkers on both sides. In RapR-p38, iFKBP was flanked by PE and NP linkers at the N and C temini, respectively. The FRB domain of human FRAP1 protein was cloned into pmCherry-CI vector using EcoRI/BamHI cloning sites. GFP-tagged FAK variants were created by subcloning the FAK gene into pEGFP-CI vector (Clontech) using BglII/BamHI cloning sites. The myc-tagged Src gene was constructed by insertion of a myc-tag sequence at the 3′-end of the Src gene using the QuikChange mutagenesis kit.

Immunoprecipitation and kinase assay.

Cells expressing FAK or Src were treated with either rapamycin or equivalent volumes of ethanol (solvent control). After treatment, cells were washed with ice-cold PBS and lysed with lysis buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 100 mM NaCl, 1 mM EGTA, 1% NP40, 1 mM NaF, 0.1 mM Na3VO4, 0.033% ethanol). Cells treated with rapamycin were lysed with lysis buffer containing 200 nM rapamycin. Cleared lysates were incubated for 2 h with IgG-linked agarose beads prebound with antibody used for immunoprecipitation. The beads were washed two times with wash buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 100 mM NaCl, 1 mM EGTA, 1% NP40) and two times with kinase reaction buffer (25 mM HEPES, pH7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM EGTA, 0.005% BRIJ-35). No $MnCl_2$ was used in the kinase reaction buffer for Src kinase immunoprecipitation and assay. Bead suspension (20 μl) was used in kinase assays using the N-terminal fragment of paxillin as previously described[31]. Kinase assay for p38α was performed as previously described[32].

Cell imaging.

Cells were plated on fibronectin-coated coverslips (10 mg/l fibronectin) 2 h before imaging, then transferred into L-15 imaging medium (Invitrogen) supplemented with 5% FBS. Live cell imaging was performed in an open heated chamber (Warner Instruments) using 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 were collected using a PhotometrixCoolSnap ES2 CCD camera controlled by Metamorph software. The 468 nm and 568 nm lines from an omnichrome series 43 Ar/Kr laser were used for TIRF imaging. Epifluorescence images were taken using a high-pressure mercury arc light source. Cells expressing GFP-RapRFAK constructs and mCherry-FRB were selected using epifluorescence imaging. Time-lapse movies were taken at 1 min time intervals. GFP-RapR-FAK expression level quantification and other image analysis were performed using Metamorph software.

Thermodynamics study of FKBP, FKBP deletion mutant with and without binding partners.

We performed replica exchange discrete molecular dynamics (DMD) simulations of various molecular systems to estimate the thermo-stabilities and to study the conformational dynamics of FKBP and its deletion mutant, dFKBP. Details of the DMD method and simulation protocols can be found in previous studies[24, 25]. Briefly, DMD is an efficient conformational sampling algorithm and an all-atom DMD model has been shown to fold several small proteins to their native states *ab initio*[25]. Using replica exchange DMD simulations, the folding thermodynamics of superoxide dismutase (SOD1) and its variants were computationally characterized in agreement with experiments[24]. We applied a similar method to study the folding thermodynamics and conformational dynamics of FKBP and dFKBP bound to either rapamycin or both rapamycin and FRB. The X-ray crystal structure of FKBP, FRB and rapamycin was used to set up the simulations (PDB code: 3FAP).

Model construction of chimeric kinase.

To model FAK with dFKBP insertion, we first manually positioned the dFKBP with various linkers in the proximity of insertion loci of FAK (PDB code: 2J0J) using PyMol (http://www.pymol.org/). To model the relative orientation of iFKBP with respect to FAK, we performed all-atom DMD simulations at 27 °C (ref. 25) with the FAK molecule kept static, whereas dFKBP and linkers were allowed to move. As the simulation temperature is below the folding transition temperature of dFKBP, the inserted domain stays folded while the DMD simulation optimizes its relative orientation with respect to FAK. By clustering the snapshot conformations from equilibrium DMD simulations, the centroid structure was identified. We modeled the chimera in complex with rapamycin and FRB by aligning the corresponding FKBP domains in the chimera and in the complex structure of FKBP, rapamycin and FRB. Similarly, we also constructed the model of FKBP insertion into Src kinase (PDB code: 1Y57).

DMD simulations of chimeric kinases.

We performed equilibrium DMD simulations of FKBP-dFKBP chimera with different linkers at 27 °C. We also studied wild-type FAK, FAK-dFKBP chimera and FAK-dFKBP chimera in complex with rapamycin and FRB. To reduce the computational overhead, we kept the distal FERM domain of FAK and alpha-helical subdomain of the catalytic domain fixed. We allowed the inserted FKBP and the directly modified catalytic subdomain to sample their conformational space. Similarly, we also studied the Src-dFKBP chimera.

The dynamic coupling of the wild-type FAK was obtained by computing the normalized correlation matrix[33, 34] from DMD simulation trajectories. In the calculation of the dynamics coupling and RMSF, the translational and rotational freedom was reduced by translating the center of mass to the origin and then aligning each snapshot with respect to the average structure.

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