SUPPLEMENT MATERIAL

FRNK Inhibition of FAK-dependent Signaling and Migration In Vascular Smooth Muscle Cells

Yevgeniya E. Koshman, Taehoon Kim, Miensheng Chu, Steven J. Engman, Rekha Iyengar, Seth L. Robia, and Allen M. Samarel

SUPPLEMENTAL METHODS

Materials and reagents. Tissue culture dishes and Permanox® chamberslides were from Nunc (Naperville, IL). Dulbecco's Modified Eagle Medium (DMEM) was from Life Technologies (Grand Island, NY). Heat-inactivated fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). Human recombinant PDGF-BB was from Calbiochem (La Jolla, CA). Rabbit polyclonal anti-FAK (pY397), anti-FAK (pY861), anti-FAK (pY925), and anti-paxillin (pY118) phosphospecific antibodies were from BioSource International (Camarillo, CA). Phospho-ERK1/2 polyclonal antibody (pT183/pY185) was from Promega (Madison, WI). GFP antibody was from Stressgen (Ann Arbor, MI). Anti-paxillin and anti-N-terminal FAK mouse monoclonal antibodies (mAb) were from BD Transduction Laboratories (Lexington, KY). Anti-GAPDH mouse mAb was purchased from Novus Biologicals (Littleton, CO). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were from BioRad (Hercules, CA). Rhodamine-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Invitrogen (Carlsbad, CA). Rainbow molecular weight standards and Enhanced Chemiluminescence (ECL) kits were from Amersham (Arlington Heights, IL). Costar transwell polystyrene plates were from Corning Costar (Cambridge, MA). All other reagents were of the highest grade commercially available and were obtained from Sigma Chemical (St. Louis, MO).

Cell culture. RASM were isolated as previously described[1] and maintained in DMEM containing 10% FBS. Cells up to the 9th passage were used.

Adenoviral constructs. Replication-defective adenoviruses (Adv) expressing GFP and GFP-wtFRNK were generated as previously described.[2] An Adv expressing GFP-L341S-FRNK was generated from a pEGFP-wtFRNK expression plasmid[3] and mounted in an Adv using the Ad-Easy XL System (Agilent Technologies, Santa Clara, CA). The multiplicity of viral infection (moi) was determined by dilution assay in HEK293 cells grown in 96-well clusters. RASM were growth-arrested in serum-free culture medium for at least 1h prior to infection. Cells were incubated (24h, 37^{o}C) with Adv in serum-free medium, and the medium was replaced with serum-free DMEM for an additional 24h.

Total internal reflection fluorescence (TIRF)-microscopy and fluorescence recovery after photobleaching (FRAP). RASM grown on 1mm glass coverslips were infected (24h, 100moi) with Adv-GFP, Adv-GFP-wtFRNK, or Adv-GFP-L341S-FRNK. Cells were then viewed under an inverted TIRF-microscope. TIRF-illumination was performed by a 445nm diode laser and/or multiline 300mW Argon laser via a Nikon TIRF II illuminator coupled with a custom beam combiner launch. For FRAP, the Argon laser was directed to the sample with a 10/90 beam splitter mounted in a second filter cube carousel positioned above the excitation dichroic. Bleach spot sizing was accomplished with a Keplerian telescope mounted on an XYZ translation stage. Laser routing and photobleaching exposure time were controlled by Uniblitz shutters driven with a shutter/filter wheel controller interfaced to a computer. A single peripheral FA in an individual cell (20 cells per group) was randomly selected, and subjected to FRAP analysis. Fluorescence intensity data were acquired every 22.9 msec for up to 20 sec after photobleaching a small region of interest encompassing a single FA site. Fluorescence intensity data immediately after the laser flash (F) were then normalized to the initial

fluorescence intensity (F_0) and plotted as a function of time (sec). Data were then fit to the following double-exponential function:

$$F/F_0 = Y_0 + M1*(1-e^{k1*t}) + M2*(1-e^{k2*t})$$

where Y_0 was the best-fitting value for F/F_0 at t=0 (i.e., immediately after the flash), M1 and M2 were the fraction of total fluorescence in the "fast" and "slow" compartments, respectively, and k1 and k2 were the first-order rate constants (sec^{-1}) describing the rate of rise of F/F_0 in each of the two compartments. Unweighted data were fit using SigmaStat Ver. 3.1 software. Best-fitting values for M1, k1, M2 and k2 were then used to calculate a single "average" rate constant for each cell (k_{FRAP}, sec^{-1}) describing the rate of rise of F/F_0 to plateau:

$$\text{Average } k_{FRAP} = M1*k1 + M2*k2$$

Confocal microscopy. RASM grown on Permonox chamberslides were infected with Adv-GFP, Adv-GFP-wtFRNK, or Adv-GFP-L341S-FRNK (100moi, 48h). Cells were then fixed, permeablized and counterstained with anti-paxillin mAb as previously described.[2] Optical sections (~1µm) at the cell-substratum interface were imaged using a Leica TCS SP5 Multiphoton Confocal Microscope.

Co-immunoprecipitation, SDS-PAGE and Western blotting. For co-immunoprecipitation experiments, RASM were scraped in homogenization buffer (25mM HEPES, pH 7.4, containing 150mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 10mM sodium pyrophosphate, 10mM NaF, 0.1mM sodium orthovanadate, 0.5% Nonidet P-40, and 1% sodium deoxycholate). Following centrifugation (10,000g, 20min), protein concentration was determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford IL) using bovine serum albumin as standard. Equal amounts of extracted cellular proteins (500µg) were immunoprecipitated with either

nonimmune IgG or anti-paxillin mAb overnight at 4°C followed by the addition of protein A/G beads (2h, 4°C) and centrifugation (10,000g; 10min). The complexes were then washed and resuspended in electrophoresis sample buffer, and separated by SDS-PAGE on 10% polyacrylamide gels. For Western blotting experiments, cells were homogenized in lysis buffer[4] and equal amounts of extracted proteins (50µg) were directly separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose, and the membranes were probed with the appropriate phosphospecific and total anti-FAK, anti-paxillin, and anti-GFP antibodies. In some experiments, equal loading was confirmed by quantifying GAPDH in each sample. Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody, and visualized by ECL.

RASM cell migration assay. Matrigel transwell migration assays were performed as previously described.[3]

Subcellular fractionation. RASM were scraped in digitonin extraction buffer (0.015% digitonin, 100mM PMSF, 2mM EDTA, 2mM EGTA, 10µg/mL aprotinin, 10µg/mL leupeptin, 500µM sodium orthovanadate and 1mM PefaBloc) and centrifuged (480g, 10min). The supernatant, soluble fraction (SF) was carefully removed. The insoluble, particulate fraction (IF) was resuspended in a buffer containing 1% Triton X-100 and 0.1% SDS, sonicated, and recentrifuged (10,000g, 20min). The SF and IF were then resuspended in lysis buffer, and equal amounts of protein from each fraction were separated by SDS-PAGE and Western blotting.

SUPPLEMENTAL REFERENCES

1. Sabri A, Govindarajan G, Griffin TM, Byron KL, Samarel AM, Lucchesi PA. Calcium- and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. *Circ Res* 1998;83:841-851.
2. Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM. GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circ Res* 2002;90:1282-1289.
3. Koshman YE, Engman SJ, Kim T, Iyengar R, Henderson KK, Samarel AM. Role of FRNK tyrosine phosphorylation in vascular smooth muscle spreading and migration. *Cardiovasc Res* 2010;85:571-581.
4. Schlaepfer DD, Hunter T. Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* 1996;16:5623-5633.

Supplemental Data

Table I. Summary data of TIRF-FRAP imaging experiments and Average k_{FRAP}.

	GFP (n=20)	wtFRNK (n=20)	L341S-FRNK (n=20)
Starting Fluorescence (Arbitrary Units)	941±108	248±16*	208±15*,+
% Photobleached	35.7±1.4	38.4±2.0*	31.2±2.5*,+
Fluorescence Recovery (%)	97.4±0.4	86.5±1.1*	92.6±1.0*,+
Average k_{FRAP} (sec^{-1})	2.55±0.22	0.427±0.057*	0.730±0.077*,+

Data are mean±SEM; *P* values were obtained by 1-way ANOVA or 1-way ANOVA on Ranks followed by Student-Newman-Keuls test, where appropriate. *P<0.05 vs. GFP; ^{+}P<0.05 vs. wtFRNK.

Supplementary Data

Table II. Comparison of Best-Fitting Parameters for wtFRNK vs. L341S-FRNK TIRF-FRAP experiments.

Curve Fitting Data:	$F/F_0=Y_0+M1*(1-e^{-k1*t})+M2*(1-e^{-k2*t})$	
	wtFRNK (n=20)	L341S-FRNK (n=20)
Y_0 (%)	61.9±2.5	69.6±1.9*
M1 (%)	33.0±4.7	43.0±4.0
k1 (sec^{-1})	1.28±0.25	1.48±0.13
M2 (%)	67.0±4.7	57.0±4.0
k2 (sec^{-1})	0.137±0.018	0.203±0.021*
Adjusted R^2	0.967±0.008	0.952±0.005

Data are mean±SEM; *P* values were obtained by Students t-test or Mann-Whitney Rank Sum test, where appropriate. *$P<0.05$ vs. wtFRNK.

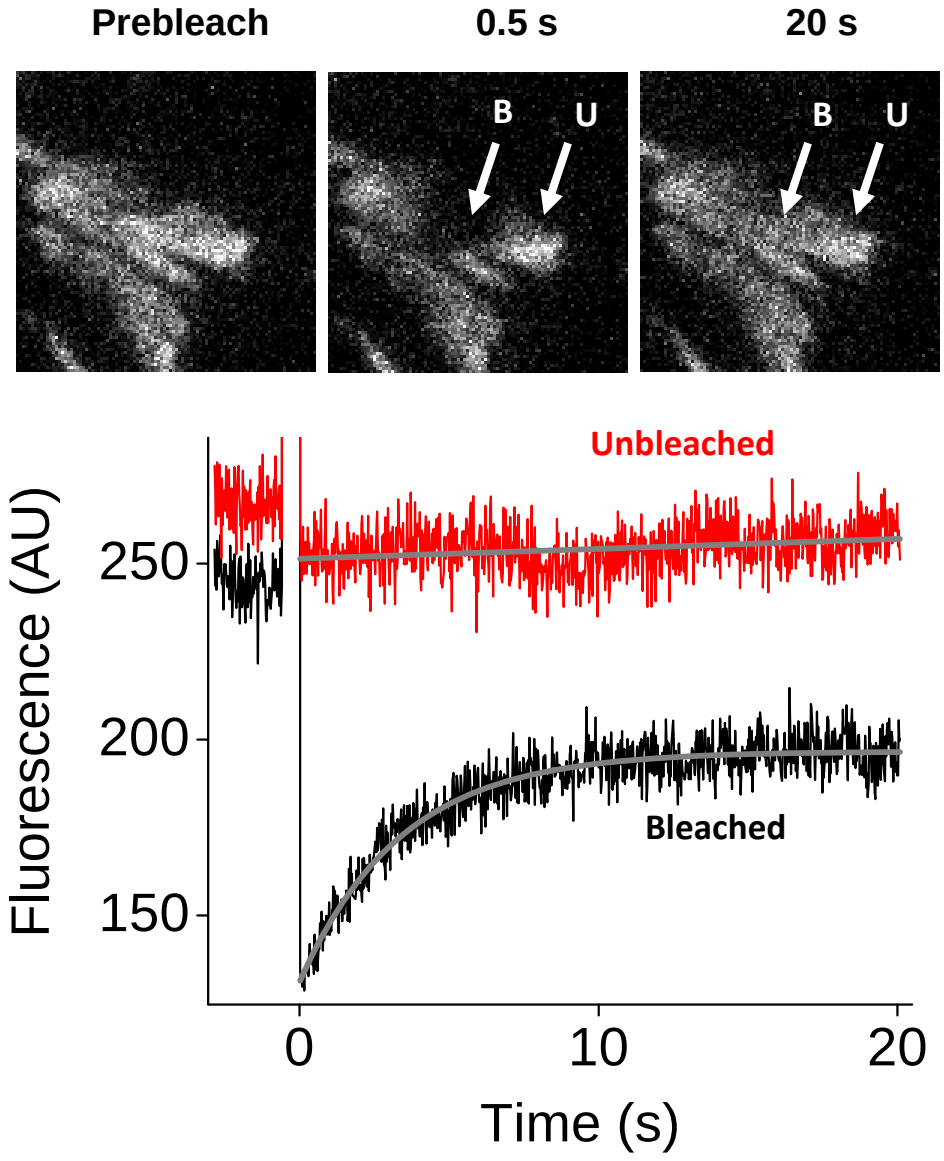

Figure I. *Rapid diffusion of GFP-FRNK from the cytoplasm vs. adjacent focal adhesion structures.* To analyze whether fluorescence recovery of GFP-wtFRNK or GFP-L341S-FRNK occurred from adjacent fluorescent proteins within same FA of an individual TIRF-FRAP experiment, we analyzed the fluorescence intensity within the bleached ROI (B), and compared it to an adjacent, unbleached region within the same FA (U). As is evident from this analysis, the bleached region (B) recovered its fluorescence without a loss of intensity in the adjacent unbleached region (U), suggesting that the ROI fluorescence was restored from a rapidly diffusible, cytoplasmic pool.