Supporting Information

Belkina et al. 10.1073/pnas.0805963106

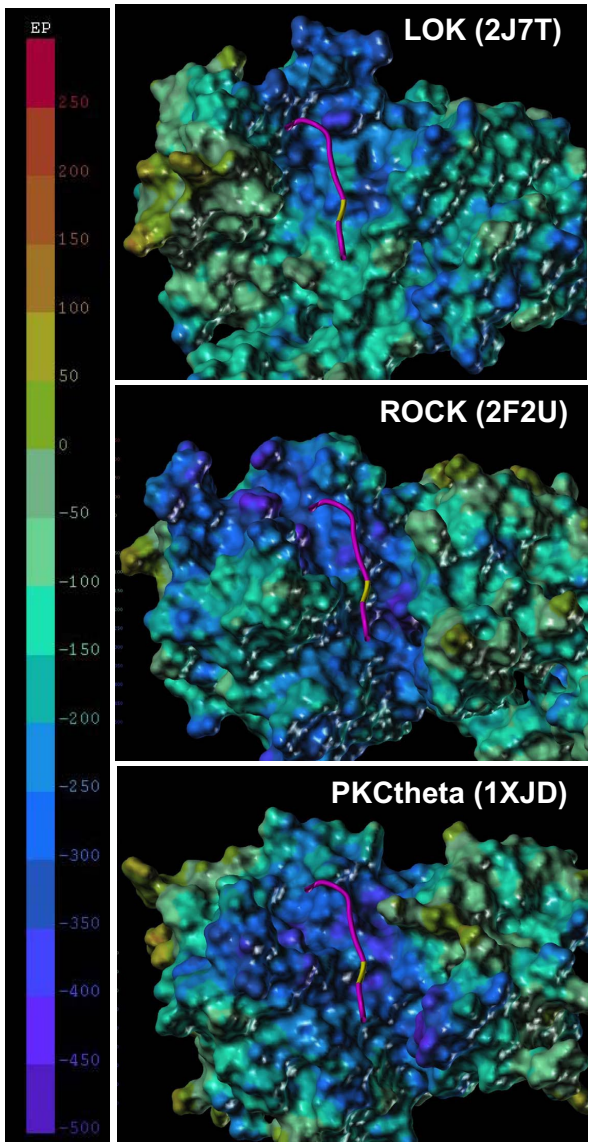

Fig. S1. Structural evidence predicting that LOK is a basophilic kinase. Modeling was done as described below. Results show that the distribution of negatively charged residues in the substrate binding cleft of LOK is suitable to accommodate basic substrate and partially resembles distribution in ROCK and PKC-θ substrate binding clefts. Solved structure of kinase domain for LOK, ROCK, and PKC-θ were obtained from PDB [ID codes 2J7T, 2F2U (1), and 1XJD (2)]. Using SYLBYL (3), ATP and substrate positions were modeled on the basis of structure of PKA in complex with peptide inhibitor (4) (using peptide truncated to P–5 through P+2). Surface electrostatic potentials were rendered using MOLCAD with AMBER7 FF99 charge set for protein and Gasteiger-Huckel method for ATP. Kinase domains are viewed from the same direction: C-lobe at the *Right* side, N-lobe at the *Left* side, P–5 of the substrate on the *Top*, P+2 on the *Bottom*. Substrate is shown as a purple tube with phosphorylation site P0 highlighted in yellow. Negative charged surface shown in blue, positive in red (the converse of those in Fig. 2*B*).

1. Yamaguchi H, Kasa M, Amano M, Kaibuchi K, Hakoshima T (2006) Molecular mechanism for the regulation of rho-kinase by dimerization and its inhibition by fasudil. *Structure* 14(3):589–600.
2. Xu ZB, et al. (2004) Catalytic domain crystal structure of protein kinase C-theta (PKCtheta). *J Biol Chem* 279(48):50401–50409.
3. SYBYL 7.0 (Tripos, Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144).
4. Zheng J, et al. (1993) 2.2 A refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MnATP and a peptide inhibitor. *Acta Crystallogr D Biol Crystallogr* 49(Pt 3):362–365.

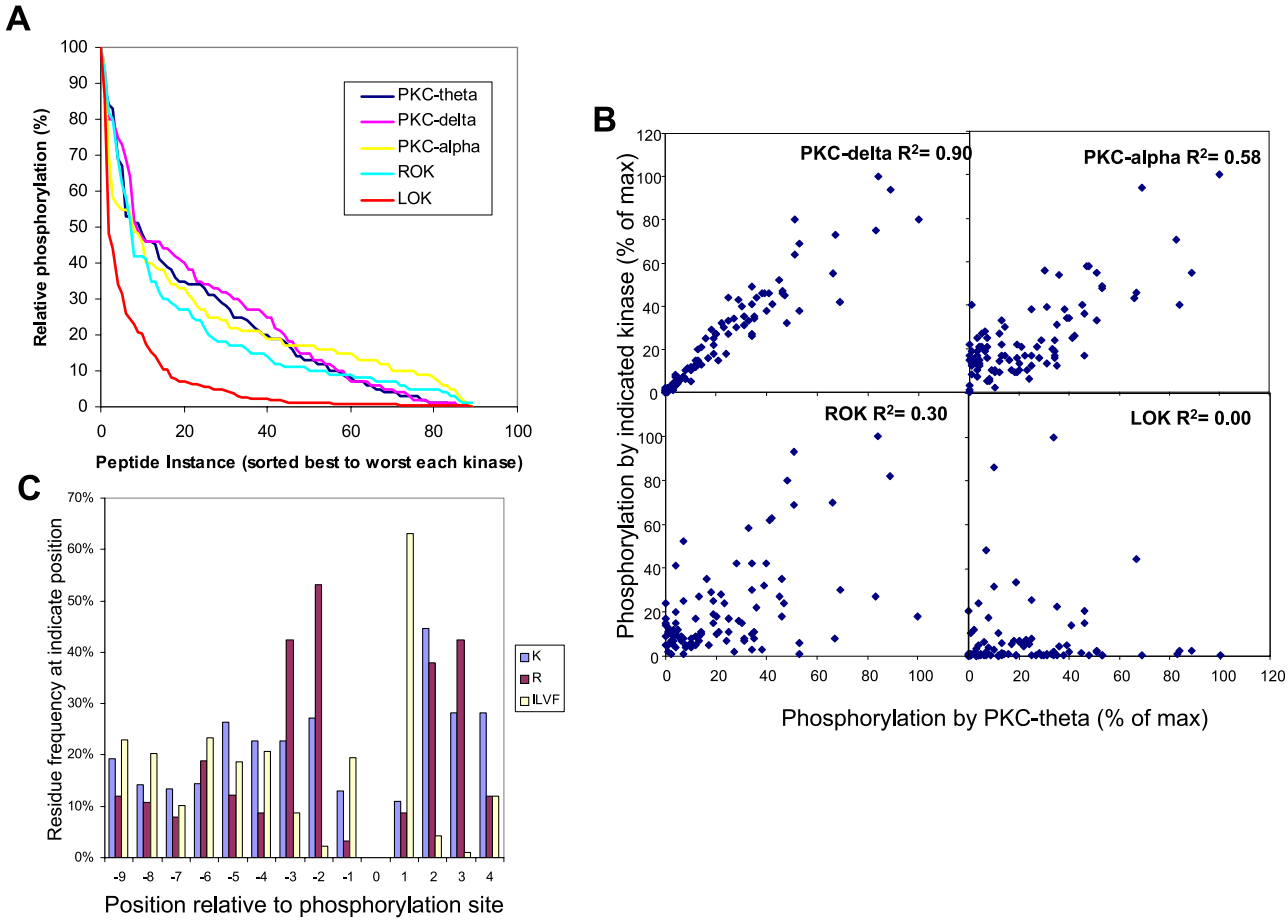

Fig. S2. LOK's specificity diverges from ROCK and PKC on the basis of phosphorylation of a panel of basic peptides from the human proteome. A panel of 90 basic peptides from the human proteome (see panel *C*) was phosphorylated in vitro by 5 kinases (PKC-θ, -δ, -α, ROK, and LOK) at substrate concentration of 10 μM as described in *Materials and Methods*. Phosphorylation of each substrate by a given kinase was expressed as [phosphorylation of that substrate]/[phosphorylation of the best substrate by that kinase]. Results represent the average from duplicate wells. (*A*) LOK efficiently phosphorylates a lower percentage of basic peptides in this panel than PKCs and ROCK. For each kinase the phosphorylation data for individual peptides is sorted in descending order. For example, for LOK, only 17 peptides achieve 10% of the level seen on the best peptide; in contrast 55 peptides (or more) were phosphorylated to that level by other kinases. Thus, although a few peptides are well phosphorylated by LOK, LOK phosphorylates a narrower spectrum of these peptides than PKC or LOK. Note that, although this peptide panel was created long before we knew that LOK is an ERM kinase, its diversity resulted in inclusion of a few good substrates (i.e., moesin peptide phosphorylation is only about 2 times better than the best in the panel). (*B*) LOK's pattern of phosphorylation of individual peptides is discordant from other kinases. Each panel is a scatterplot in which each dot represents a peptide whose placement in the panel is determined by its phosphorylation by PKC-θ (*x*-axis) and kinase indicated in the panel (*y*-axis). The Pearson correlation coefficient for the values is as listed. (*C*) The peptides were synthesized by Axcell Biosciences and in vitro kinase assays were conducted as previously described (1). The peptides generally contained 15 residues N-terminal to an S/T and 4 residues C-terminal. All sequences were selected from the human proteome using the general objectives of enriching for peptide characteristics that are favorable for many conventional basophilic kinases : (*i*) several R or K at positions P–3, P–2, P+2, P+3; (*ii*) hydrophobic residue I/L/V/F at P+1; and (*iii*) apparently expressed in human lymphocytes. The residue distribution for peptides in the set are shown in panel *C*.

1. Fujii K, et al. (2004) Kinase peptide specificity: improved determination and relevance to protein phosphorylation. *Proc Natl Acad Sci USA* 101(38):13744–13749.

Km (uM)	Moesin	Moesin Y556R
LOK	13.5+/-4.6	21.1+/-8.6
ROCK	6.8+/-3.1	6.3+/-2.0
PKC	4.4+/-0.5	10.8+/-1.6

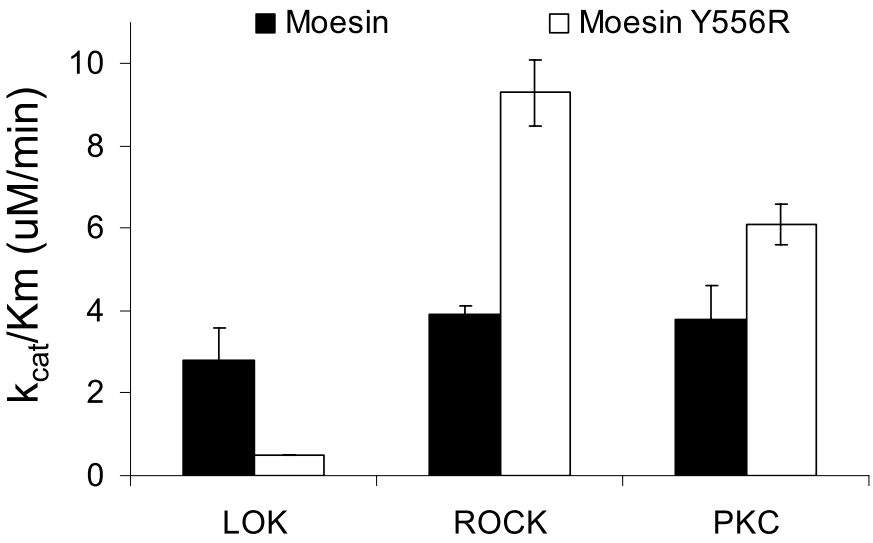

Fig. S3. LOK has distinctive specificity for the Y at P–2 of the ERM C-terminal phosphorylation site. Kinetic analysis of in vitro phosphorylation by LOK, ROCK, and PKC-θ of 2 peptides: WT moesin and mutant peptide where Y at P–2 is substituted with R. For kcat values see Fig 2*D*. Note that ERM concentration in lymphocytes is very high, especially near the membrane (1), the modestly higher K_m of LOK for moesin peptide is not likely to be an important limitation in the physiologic context. The assay was done as described in *Materials and Methods*.

1. Hao JJ, et al. (2008) Enrichment of distinct microfilament-associated and GTP-binding proteins in membrane/microvilli fractions from lymphoid cells. *J Proteome Res* 7 (7):2911–2927. See *SI Text* spreadsheet http://pubs.acs.org/doi/suppl/10.1021/pr800016a/suppl_file/pr800016a-file004.xls)

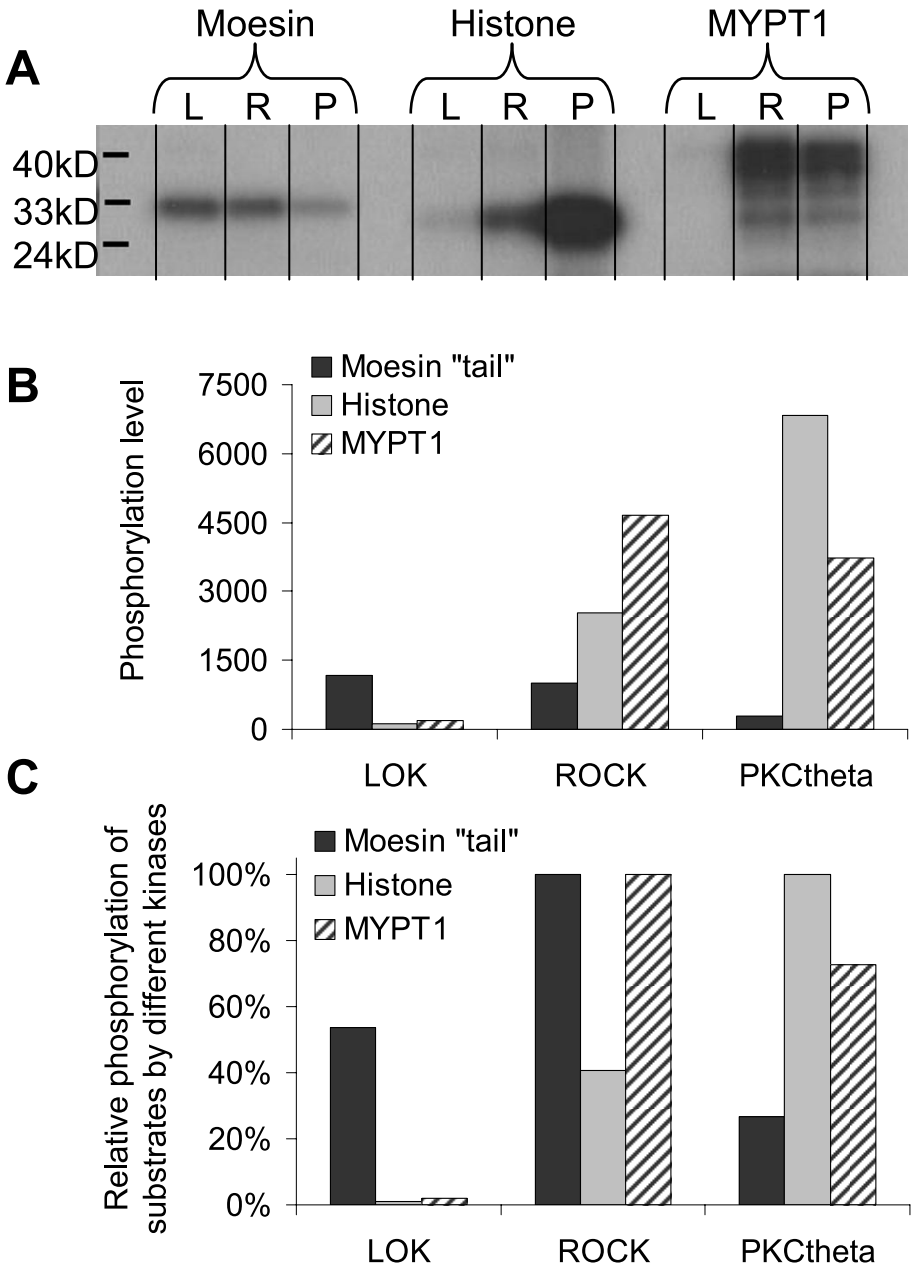

Fig. S4. LOK has distinctive preference for moesin compared to 2 conventional basic protein substrates. (*A*) Results of in vitro kinase assay of phosphorylation of 3 protein substrates moesin C terminus (302–577), histone f1 fraction, and MYPT1 (654–880) by LOK (L), ROCK (R), and PKC-θ (P). Autoradiogram is shown of ^{32}P incorporation into products resolved on PAGE. (*B*) Quantitation of results shown at panel *A* by densitometry. (*C*) Relative preference of LOK, ROCK, and PKC-θ for 3 basic protein substrates. Phosphorylation may occur at multiple sites, since the readout is total ^{32}P incorporation. To adjust phosphorylation to equal kinase molarities, densitometric readings from panel *A* are adjusted by the relative molar concentrations of each kinase (approximately 2 times in excess of PKC-θ and ROK). Phosphorylation of each substrate is then expressed relative to its phosphorylation by the kinase most active on it. Protein phosphorylation was done as described in *Materials and Methods*, except that 10 μCi of γ^{32}P-ATP, 4 ng of protein substrate and 50 ng of kinase were used in total 50 μL volume for 1 h. Reaction was stopped by adding 2$\times$ reducing SDS sample buffer and samples were separated as described for Western blot. The ^{32}P emission from the gel then was acquired as described for streptavidin-coated membrane.

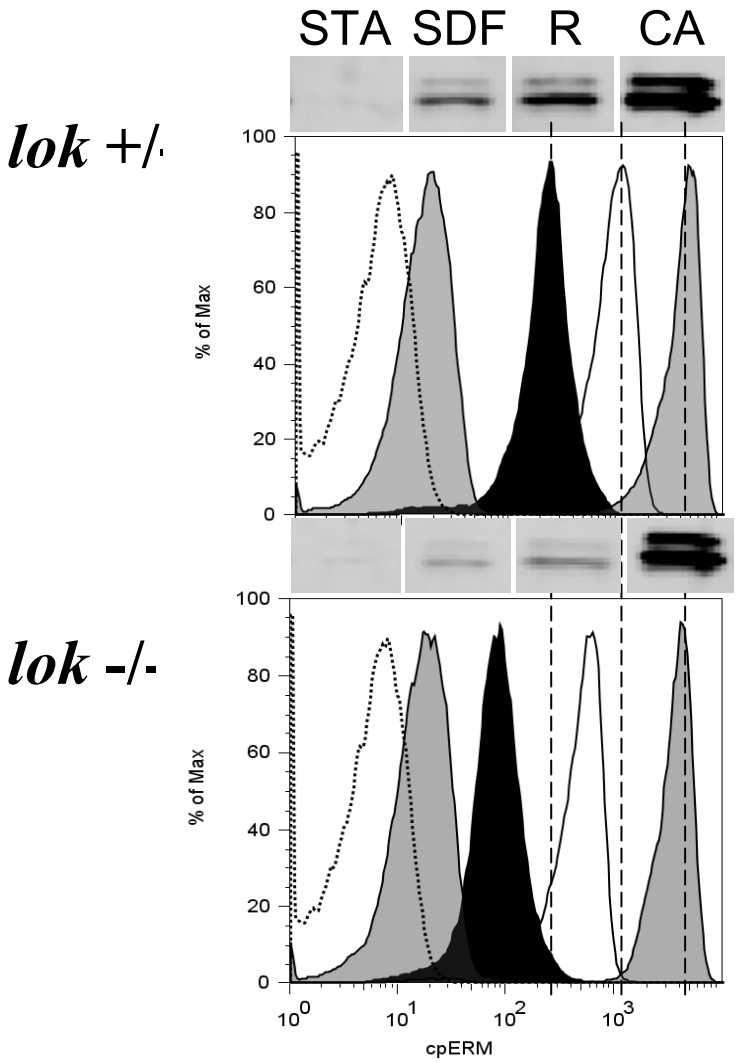

Fig. S5. Comparison of cpERM level in LOK-knockout and littermate mice by Western blot and flow cytometry. cpERM was assessed both by Western blot (*Upper* subpanel) and flow cytometry (*Lower* subpanel). Splenic lymphocytes from LOK-knockout mice and littermate controls were analyzed under 4 standard conditions: R: resting cells, no treatment, solid line; SDF-1, 1 min after 100 ng/mL SDF-1 to assess physiological dephosphorylation, (black fill); STA, 1 min after high concentration (500 nM) staurosporine to assess maximal dephosphorylation, (gray fill on *Left*); CA, 5 min after phosphatase inhibitor 50 μM calyculin A to maximize phosphorylation, (gray fill on far *Right*); (for flow cytometry, unfilled curve on far *Left* is the no-antibody control)

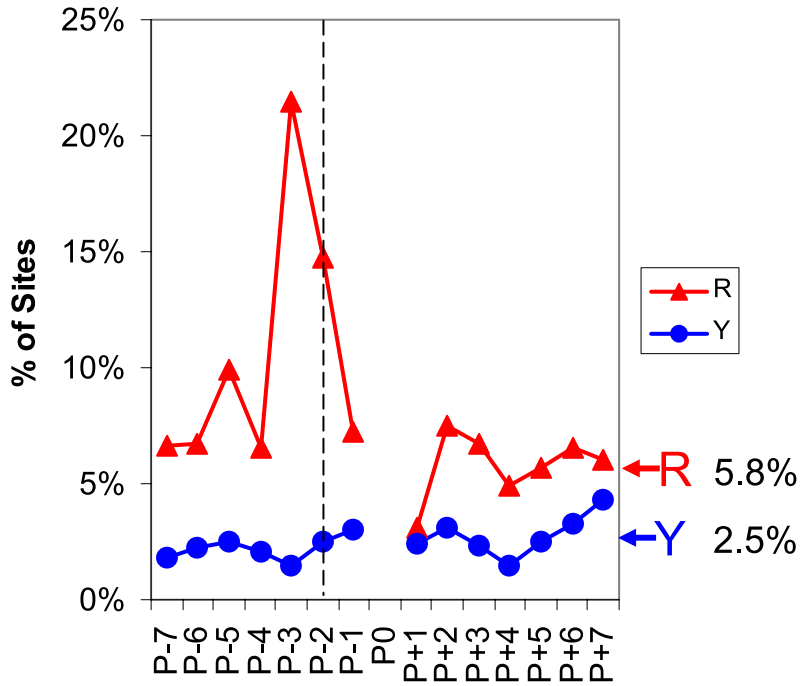

Fig. S6. Arg is overrepresented at P–2 among known phosphorylation sites but Tyr is not. Residue abundance was determined at positions around known phosphorylation sites. We have previously reported results for basic residue frequency from this analysis (1). Data shown are extracted by analysis of sites curated by Phosphosite (http://phosphosite.cellsignal.com/) a publicly available resource from Cell Signaling Technology, whose curation process annotates from the literature as comprehensively as possible in vivo phosphorylation sites of mammalian proteins. We eliminated the 2 sources of site overcounting by restricting the list to human sites (i.e., excluding corresponding sites in mouse and rat), and removing redundant sites because of different isoforms of a single gene. After cleanup, 1,441 human Ser/Thr phosphorylation sites remained. Values at *Lower Right* indicate the overall frequency of R and Y in the human proteome.

1. Zhu G, et al. (2005) A single pair of acidic residues in the kinase major groove mediates strong substrate preference for P–2 or P–5 arginine in the AGC, CAMK, and STE kinase families. *J Biol Chem* 280(43):36372–36379.