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online data

SUPPLEMENTARY ONLINE DATA

Development of an intracellularly acting inhibitory peptide selective for PKN

Kazuhiro SHIGA*, Kentaro TAKAYAMA†, Shiroh FUTAKI†, Jessica E. HUTTI‡, Lewis C. CANTLEY‡, Katsuko UEKI§, Yoshitaka ONO*‖ and Hideyuki MUKAI*‖[1]

*Graduate School of Science, Kobe University, Kobe 657-8501, Japan, †Institute for Chemical Research, Kyoto University, Kyoto 611-0011, Japan, ‡Department of Systems Biology, Harvard Medical School, Boston, MA 02215, U.S.A., §Graduate School of Science and Technology, Kobe University, Kobe 657-8501, Japan, and ‖Biosignal Research Center, Kobe University, Kobe 657-8501, Japan

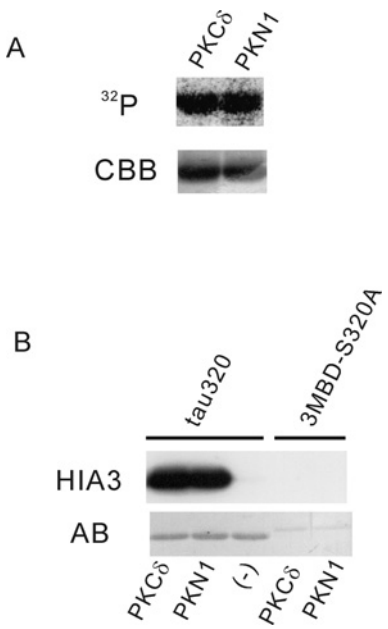

Figure S1 Phosphorylation of GST-tagged tau320 by PKN and PKC

The tau320 peptide (see Table 1 of the main text) was fused to GST and subjected to an *in vitro* kinase assay by PKN1 and PKCδ. (**A**) ^{32}P, autoradiography; CBB, Coomassie Brilliant Blue staining. (**B**) Immunoblotting using a phosphospecific antibody against Ser^{320} of tau protein (HIA3). GST-tagged microtubule binding domain (MBD) of tau with an S320A mutation (3MBD-S320A; [1]) was used as a negative control for the immunoblotting. tau320, GST-tagged tau320 peptide; AB, Amido Black staining of PVDF membrane-bound proteins; (−), enzyme free.

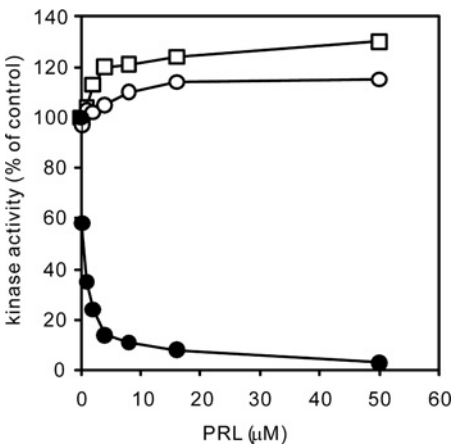

Figure S2 The effect of PRL on the *in vitro* kinase activity of Rho-kinase (ROCK2/ROKα) and PKA

The Myc-tagged catalytic domain of Rho-kinase was immunoprecipitated with 9E10 antibody from COS7 cells and was resuspended in a buffer containing 20 mM Tris/HCl at pH 7.5, and 4 mM $MgCl_2$ (pEF-BOS-myc-Rho-kinase CAT vector was kindly provided by Dr Kaibuchi, Nagoya University, Nagoya, Japan). Aliquots were then incubated for 5 min at 30°C in a reaction mixture (25 μl) containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 40 μM ATP, 18.5 kBq of [γ-^{32}P]ATP, 100 μM S6 peptide as the phosphate acceptor, and the indicated concentration of the PRL peptide. The kinase activity of PKA (kindly provided by Dr Kuno, Kobe University, Kobe, Japan) was measured by incubating the catalytic subunit of the enzyme (10 ng) for 5 min at 30°C in a reaction mixture containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 40 μM ATP, 18.5 kBq of [γ-^{32}P] ATP, 0.1 mg/ml recombinant GST as the stabilizer, 40 μM Kemptide as the phosphate acceptor, and the indicated concentration of the PRL peptide. Reactions were terminated by spotting them on to Whatman P81 phosphocellulose papers, submersing them in 75 mM phosphate, and then washing three times for 10 min. The incorporation of [^{32}P]phosphate into the S6 peptide (for Rho-kinase) or Kemptide (for PKA) was assessed by liquid-scintillation counting. PKA (□), Rho-kinase (○) and PKN1 (●).

[1] To whom correspondence should be addressed (email mukinase@kobe-u.ac.jp).

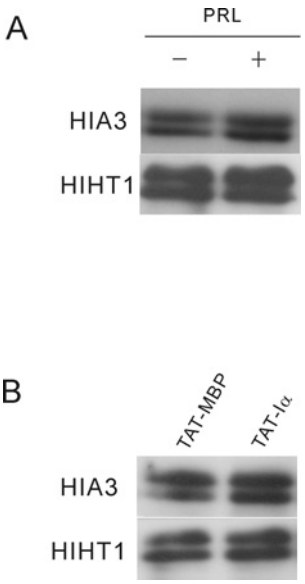

Figure S3 The treatment of HeLa cells with unmodified PRL and TAT-tagged Iα

HeLa cells were transfected with pSG5/tau in combination with pTB701/PKN1/AF3 (aPKN). The phosphorylation level was visualized using a phosphospecific antibody against Ser^{320} of tau protein (HIA3). The total amount of tau protein irrespective of its phosphorylation state was visualized using antibody HIHT1. (**A**) The effect of the treatment with the unmodified PRL peptide on Ser^{320} phosphorylation. HeLa cells, expressing recombinant tau and aPKN, were treated with or without 100 μM unmodified PRL peptide for 20 min. (**B**) The effect of TAT-tagged Iα on Ser^{320} phosphorylation. HeLa cells, expressing recombinant tau and aPKN, were treated with 10 μM TAT-tagged Iα (TAT-Iα) or 10 μM TAT-tagged myelin basic protein (TAT-MBP) as a control for 20 min.

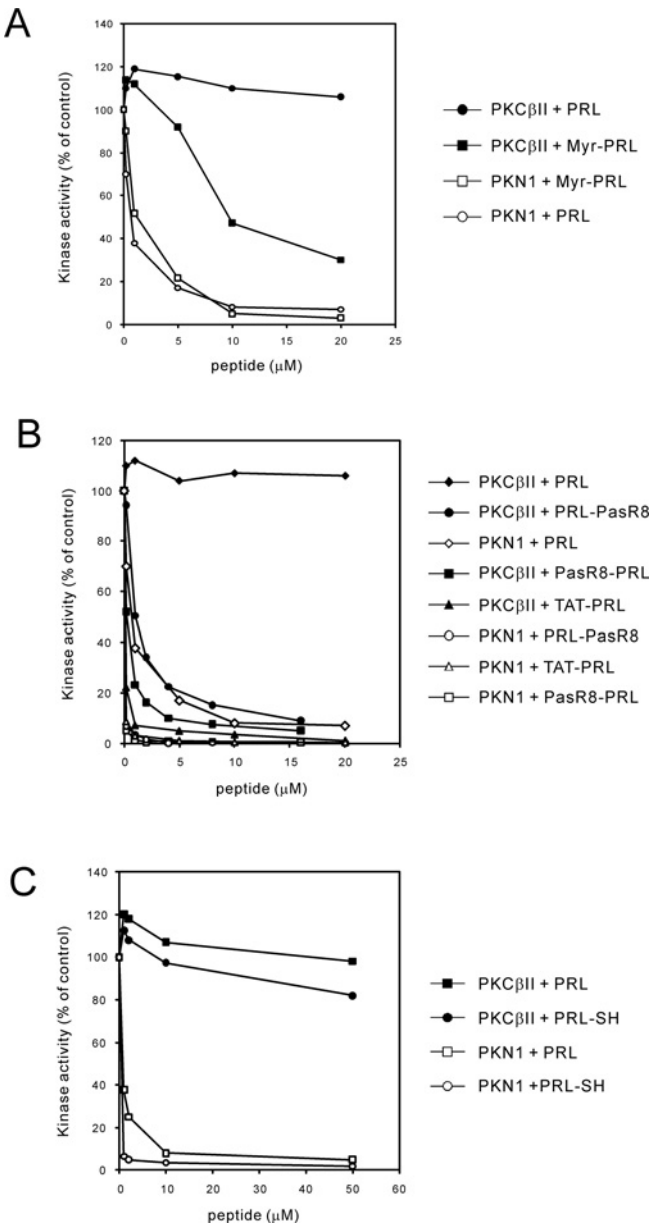

Figure S4 The effect of modified PRL on the *in vitro* kinase activity of PKN and PKC

The activity of the catalytic domain of PKN1 and PKCβII was assayed for δpeptide in the presence of various concentrations of the modified or unmodified PRL peptides. The δpeptide kinase activity of the enzyme in the absence of additional peptides was taken as 100 % activity. The result shown is representative of three independent experiments. (**A**) The effect of myristoylated PRL on the kinase activity. Myr-PRL, the myristoylated PRL peptide. (**B**) The effect of CPP-tagged PRL on the kinase activity. (**C**) The effect of PRL-SH on the kinase activity.

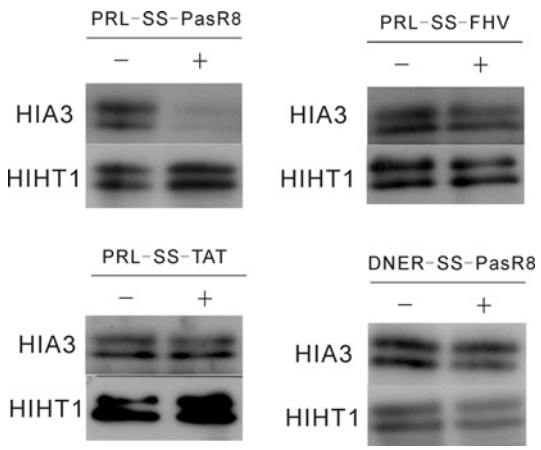

Figure S5 The effect of PRL-SS-CPPs on the *in vivo* kinase activity of PKN1 in CHO-K1 cells

CHO-K1 cells were transfected with pSG5/tau in combination with pTB701/PKN1/AF3 (aPKN). The phosphorylation level was visualized using a phosphospecific antibody against Ser^{320} of tau protein (HIA3). The total amount of tau protein irrespective of its phosphorylation state was visualized using antibody HIHT1. CHO-K1 cells, expressing recombinant tau and aPKN, were treated with or without 10 μM PRL-SS-PasR8, PRL-SS-FHV, PRL-SS-TAT, and control DNER-SS-PasR8 peptide for 20 min.

REFERENCE

1 Taniguchi, T., Kawamata, T., Mukai, H., Hasegawa, H., Isagawa, T., Yasuda, M., Hashimoto, T., Terashima, A., Nakai, M., Mori, H. et al. (2001) Phosphorylation of tau is regulated by PKN. J. Biol. Chem. **276**, 10025–10031

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