Protein–Protein Interactions

DOI: 10.1002/anie.201005333

Direct Monitoring of the Inhibition of Protein–Protein Interactions in Cells by Translocation of PKCδ Fusion Proteins**

Kyung-Bok Lee, Jung Me Hwang, Insung S. Choi, Jaerang Rho, Jong-Soon Choi, Gun-Hwa Kim, Seung Il Kim, Soohyun Kim, and Zee-Won Lee**

In the field of drug discovery and development, the increasing use of cell-based assays has resulted in an increased demand for novel cellular bioassays. Such bioassays are expected to detect a wide variety of functional molecules in live cells.[1] Fluorescence-based imaging techniques such as fluorescence resonance energy transfer (FRET) and biomolecular fluorescence complementation (BiFC) have been developed to analyze protein–protein interactions (PPIs) and inhibition of PPIs (iPPIs) in live mammalian cells. Although these techniques have been useful, they require a variety of fusion constructs to determine the relative locations of fluorophores and binding pairs for optimal performance as well as appropriate linker domains.[2]

Alternatively, translocation-based cellular assays (redistribution approaches), which are cell-based assay techniques utilizing protein translocation as the primary readout, have been used to study the PPIs between specific proteins and other intracellular events.[3] These methods use a bait (target) molecule fused to a protein that changes its localization within the cell following a stimulus. Such assays can be formatted as agonist or antagonist assays, in which compounds are tested for their ability to promote or inhibit, respectively, protein translocation caused by a known agonist. Translocation-based cellular assays do not require much construct optimization and boast a high signal-to-noise ratio. These assays are robust, fast, and flexible; thus, these systems have been considered as an ideal assay for high-content-screening approaches to drug discovery.[3,4a] Despite these advantages, few experimental applications of translocation-based cellular assays have been reported. Most of these have been based on regulated transport between the cell nucleus and the cytoplasm using a combination of nuclear localization signals and/or nuclear export signals. Several technologies are already commercially available.

Recently, the groups of Schultz and Heo independently reported that PPIs can be visualized by cotranslocation of a target protein from the cytoplasm to the plasma membrane and to the endosome, respectively.[4] Schultz et al. demonstrated the direct cotranslocation of a protein complex through the Ca^{2+}-induced translocation of a bait protein fused to Annexin A4, a phospholipid- and Ca^{2+}-binding protein.[4a] Heo and colleagues showed that Rab5, an endosome-localized protein, recruited an interacting protein to the endosome through an FKBP–rapamycin–FRB complex intermediate.[4b] These studies were focused on the visual detection of PPIs so that new conceptual and novel applications of redistribution approaches have vastly expanded what can be explored in live cells.

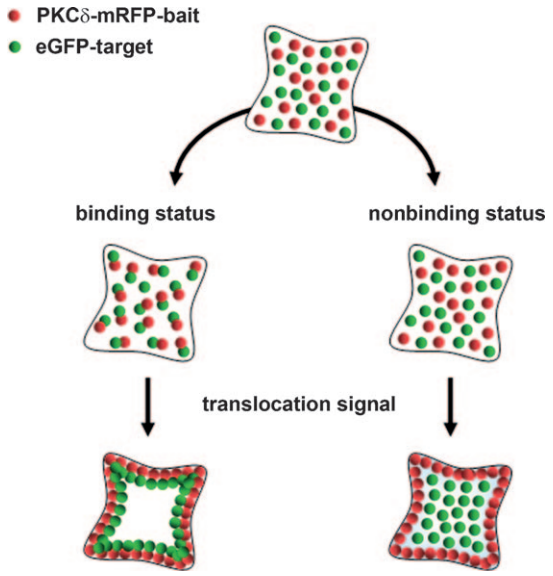
Herein we demonstrate that the inhibition of protein–protein interactions (iPPI) using a small molecular inhibitor can be monitored directly by a redistribution approach. Protein kinase C (PKC) is known to translocate from the cytoplasm to the plasma membrane in response to physiological stimuli, as well as exogenous ligands such as phorbol esters.[5] In a study using PKC tagged with green fluorescent protein (GFP) the dynamics of PKC translocation in response to different stimuli was monitored in real time in live cells.[6] PKCδ has a C1 domain that binds diacylglycerol, but an impaired C2 domain that does not bind Ca^{2+} ions. Thus, PKCδ responds to an increase in phorbol esters in the cell but not Ca^{2+} ions.[5a] Therefore we hypothesized that a PKCδ-fused bait protein would guide cotranslocation with the target protein, and a chemical inhibitor would interrupts PPI, making it possible to monitor iPPI (Scheme 1).

To verify our approach, we examined iPPI using the p53 (tumor suppressor)/MDM2 (negative regulator of the p53) protein pair[7] and Nutlin-3 (see the Supporting Information for experimental details). The small molecular inhibitor Nutlin-3 is a *cis*-imidazoline analogue commonly used in anticancer studies that inhibits the interaction between p53 and MDM2; this inhibitor resulted from the optimization of a lead structure identified by the screening of a chemical library.[8] We prepared the C-terminal fusion constructs PKCδ/monomeric red fluorescent protein (mRFP)/p53 (bait) and enhanced GFP (eGFP)/MDM2 (target). Both the pmRFP plasmid encoding PKCδ–mRFP–p53 and the peGFP plasmid encoding eGFP–MDM2 were transiently cotransfected into HEK-293T cells. When the exogenous ligand phorbol 12-myristate 13-acetate (PMA) was added, both p53

[*] Dr. K.-B. Lee,[+] J. M. Hwang,[+] Dr. J.-S. Choi, Dr. G.-H. Kim, Dr. S. I. Kim, Dr. S. Kim, Dr. Z.-W. Lee
Division of Life Science, Korea Basic Science Institute (KBSI)
Daejeon 305-333 (Korea)
Fax: (+82) 42-865-3419
E-mail: shkim@kbsi.re.kr
ezone@kbsi.re.kr
J. M. Hwang,[+] Prof. Dr. J. Rho
Department of Bioscience and Biotechnology
Chungnam National University, Daejeon 305-764 (Korea)
Prof. Dr. I. S. Choi
Department of Chemistry, KAIST
Daejeon 305-701 (Korea)
[+] These authors contributed equally to this work.

[**] This work was supported by the K-MeP project funded by the KBSI (T30130). We thank Prof. K.-M. Heo at Chungnam National University for expert technical assistance.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201005333.



Scheme 1. A schematic representation of a protein–protein interaction inhibition assay involving chemical inhibitors. A PKCδ fusion protein translocates from the cytoplasm to the plasma membrane in response to a translocation signal (in this study provided by PMA). Left: If the bait and the interacting partner (target) bind, the bait/target proteins are cotranslocated to the plasma membrane. Right: If bait and target do not bind owing to the addition of a chemical inhibitor, the target protein remains in the cytoplasm.

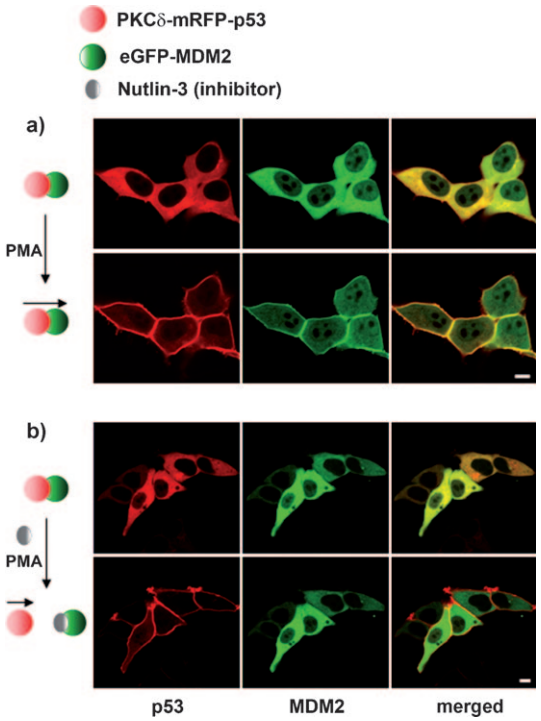


Figure 1. Confocal images of a specific PPI and iPPI in cells. a) HEK-293T cells were cotransfected with PKCδ–mRFP–p53 (bait) and eGFP–MDM2 (target). Before PMA treatment, the p53 and MDM2 proteins were localized in the cytoplasm (top row). However, after PMA (1 μM) had been added, MDM2 was cotranslocated to the plasma membrane because of the translocation property of PKCδ (bottom row). b) When cells were treated with Nutlin-3 (1 μM, inhibitor) for 30 min before PMA treatment, only the p53 protein was translocated to the plasma membrane (bottom row). The scale bar is 10 μm. See the Supporting Information for details of the imaging techniques.

and MDM2 were cotranslocated to the plasma membrane (Figure 1a bottom row; also see Movie S1 and Figure S1 in the Supporting Information). The extent and rate of cotranslocation depended on the concentration of PMA (data not shown). In contrast, Figure 1b shows that in cells treated with Nutlin-3 before PMA treatment the interaction between p53 and MDM2 was inhibited. The bottom row shows that only the p53 protein was translocated to the plasma membrane while the distribution of eGFP–MDM2 did not change (see Movie S2, Figures S1 and S2 in the Supporting Information). To evaluate the specificity of our system, we tested non-interacting protein pairs such as mRFP/eGFP, p53/eGFP, and p53/CHMP1A. The target proteins (eGFP and CHMP1A) were not translocated to the plasma membrane (see Figure S3 in the Supporting Information). These results indicate that nonspecific interactions do not occur between the PKCδ fusion construct and eGFP, nor between mRFP and eGFP.

We also found that the induction and reversal of PPIs can be detected in our assay with the FKBP (FK506 binding protein)/FRB (FKBP-rapamycin binding protein) protein pair.[9] This is an ideal system since the formation of this complex is directly induced by rapamycin and competitively inhibited by FK506, thereby ensuring that the observed complementation is driven by specific molecular interactions.[9c] The fusion constructs PKCδ–mRFP–FKBP12 and eGFP–FRB were prepared and co-expressed in cells. The cells were treated with rapamycin and/or FK506. In the absence of inducer, we observed that the FKBP12 protein was translocated to the plasma membrane (Figure 2a). As expected, treatment with rapamycin (20 nM for 10 min) induced an interaction between FKBP12 and FRB. Thus, FRB was cotranslocated to the plasma membrane (Figure 2b). Finally, the FK506-treated cells (preincubated with 20 nM of rapamycin) exhibited competitive iPPI between FKBP12–rapamycin and the FRB complex (Figure 2c and Figure S5 in the Supporting Information). These results strongly support that this assay can be used to monitor directly constitutive PPI and iPPI in live cells, and suggests potential for active compound screening of chemical libraries.

Since this approach makes it possible to visualize Nutlin-3 and FK506 iPPI in live cells, we also examined whether our assay monitors the blockage of signaling pathways. The modulation of individual components in signaling pathways has attracted a great deal of attention for the development of anticancer drugs. For example, mitogen-activated protein kinase (MAPK) cascades are key signaling pathways involved in the regulation of normal cell proliferation, survival, and differentiation. MAPKs are activated by means of a kinase cascade that results in dual phosphorylation at tyrosine and threonine residues and consequent activation.[10] Aberrant activation of the Ras/Raf/mitogen-activated ERK-activating kinase (MEK)/extracellular-regulated kinase (ERK) signaling pathway is commonly observed in a wide variety of cancers.[11]

To verify the blockage of the MAPK kinase signaling pathway, we prepared both the C-terminal PKCδ–mRFP–

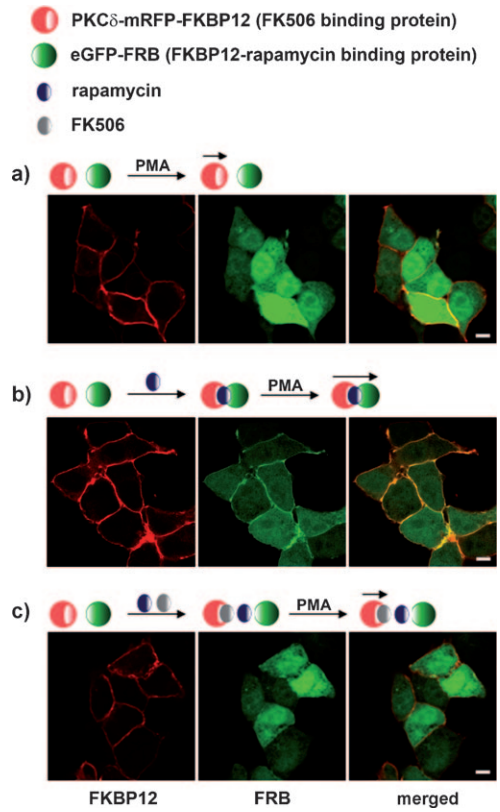


Figure 2. Confocal images of the induction and competitive inhibition of PPI in cells. HEK-293T cells were cotransfected with PKCδ–mRFP–FKBP12 and eGFP–FRB. a) In the absence of inducer, FKBP12 and FRB did not interact. b) After induction (20 nM rapamycin for 10 min), FRB was cotranslocated to the plasma membrane. c) FK506-treated cells exhibited competitive iPPI between FKBP12–rapamycin and the FRB complex (100 nM of FK506 was added for 10 min after treatment of cells with 20 nM of rapamycin for 10 min). The scale bar is 10 μm.

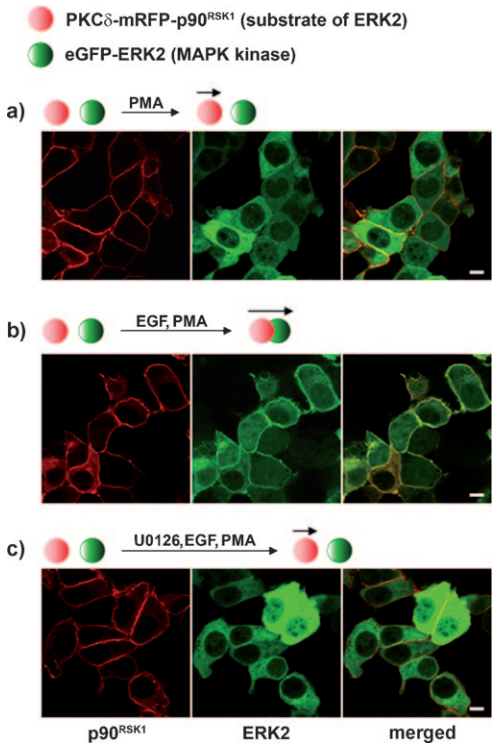


Figure 3. Confocal images of the blockage of the MEK/ERK/p90^{RSK} signaling pathway in cells. HEK-293T cells were cotransfected with PKCδ–mRFP–p90^{RSK1} and eGFP–ERK2. a) In the absence of inducer, p90^{RSK1} and ERK2 did not interact. b) After induction (100 ng mL^{-1} of EGF for 5 min), ERK2 was cotranslocated to the plasma membrane. c) Cells treated with U0126 before EGF treatment exhibited blockage of the MEK/ERK/p90^{RSK} signaling pathway between ERK2 and p90^{RSK1} (10 μM of U0126 was added to cells for 1 h before EGF treatment). The scale bar is 10 μm.

p90^{RSK1} and eGFP–ERK2 fusion constructs. The p90 ribosomal S6 kinase (p90^{RSK}) is a downstream effector of MAPK and one of the substrates of ERK1/2.[12] U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthiol)butadiene), a potent and specific inhibitor of MEK1/2, is capable of inhibiting activated MEK1/2 directly and preventing endogenously active MEK1/2 from phosphorylating and activating ERK1/2. Therefore, the compound U0126 blocks downstream MAPK signaling.[13] In the absence of serum stimuli, only the p90^{RSK1} protein was translocated to the plasma membrane (Figure 3a). In contrast, in the presence of epidermal growth factor (EGF), a serum stimulus induced the MEK/ERK/p90^{RSK} signaling pathway, resulting in the cotranslocation of ERK2 to the plasma membrane (Figure 3b). The stimulation of cells by EGF changes the levels of phosphorylation of ERK2. Thus, activated ERK2 interacts with p90^{RSK1} so that bound ERK2 can phosphorylate p90^{RSK1}.[12] Finally, U0126-pretreated cells (10 μM) exhibited the blockage of the MAPK signaling pathway between ERK2 and p90^{RSK1}. The ERK2 protein did not translocate to the plasma membrane (Figure 3c). These results show that our assay can also be used to visualize the interaction/inhibition (blockage) of individual components of signal transduction pathways.

In summary, we have developed a simple method for directly monitoring iPPI in live cells using a small molecular inhibitor. The bait protein was fused to PKCδ, which enables the bait and target proteins to cotranslocate from the cytoplasm to the plasma membrane. In contrast, when the bait/target interaction was inhibited by chemical inhibitors, only the bait protein was translocated to the plasma membrane while the distribution of target protein remained unchanged. Furthermore, we demonstrated that our assay can be expanded to test whether different sets of protein pairs interact. We verified three cases of interaction: p53/MDM2 for interaction and inhibition, FKBP/FRB for induction and competitive inhibition, and p90^{RSK1}/ERK2 for the blockage of signaling pathways. Our technique is robust and widely applicable to the analysis of novel interacting partners such as chemical compounds, peptides, and proteins for library screening.

Received: August 26, 2010
Published online: January 5, 2011

Keywords: cell-based assays · protein kinase C · protein–protein interactions

[1] a) K. A. Giuliano, D. Premkumar, D. L. Taylor in *High Content Screening: Science Technology and Applications* (Eds.: S. A. Haney), Wiley, Hoboken, **2008**, pp. 371 – 388, and references therein.

[2] a) E. A. Jares-Erijman, T. M. Jovin, *Nat. Biotechnol.* **2003**, *21*, 1387 – 1395; b) T. K. Kerppola, *Nat. Protoc.* **2006**, *1*, 1278 – 1286; c) T. K. Kerppola, *Annu. Rev. Biophys.* **2008**, *37*, 465 – 487; d) M. Fernández-Suárez, T. S. Chen, A. Y. Ting, *J. Am. Chem. Soc.* **2008**, *130*, 9251 – 9253.

[3] a) V. Fetz, S. K. Knauer, C. Bier, J. P. V. Kries, R. H. Stauber, *Sensors* **2009**, *9*, 5423 – 5445, and references therein; b) K. A. Giuliano, Y.-T. Chen, J. R. Haskins, *Mod. Drug Discovery* **2003**, *6*, 33 – 37.

[4] a) A. Piljić, C. Schultz, *ACS Chem. Biol.* **2008**, *3*, 749 – 755; b) K. H. Lee, S. Lee, W. Y. Lee, H. W. Yang, W. D. Heo, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 3412 – 3417.

[5] a) A. C. Newton in *Encyclopedia of Biological Chemistry*, *Vol. 3* (Eds.: W. J. Lennarz, M. D. Lane, P. Modrich, J. Dixon, E. Carafoli, J. Exton, D. Cleveland), Academic Press, New York, **2004**, pp. 523 – 526; b) F. Colon-Gonzalez, M. G. Kazanietz, *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2006**, *1761*, 827 – 837; c) M. A. Lemmon, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 99 – 111.

[6] a) N. Sakai, K. Sasaki, N. Ikegaki, Y. Shirai, Y. Ono, N. Saito, *J. Cell Biol.* **1997**, *139*, 1465 – 1476; b) E. Oancea, T. Meyer, *Cell* **1998**, *95*, 307 – 318; c) J. L. Baryza, S. E. Brenner, M. L. Craske, T. Meyer, P. A. Wender, *Chem. Biol.* **2004**, *11*, 1261 – 1267.

[7] U. T. Moll, O. Petrenko, *Mol. Cancer Res.* **2003**, *1*, 1001 – 1008.

[8] a) L. T. Vassilev, B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, E. A. Liu, *Science* **2004**, *303*, 844 – 848; b) L. T. Vassilev, *Trends Mol. Med.* **2007**, *13*, 23 – 31; c) S. Shangary, S. Wang, *Annu. Rev. Pharmacol. Toxicol.* **2009**, *49*, 223 – 241.

[9] a) J. Chen, X. F. Zheng, E. J. Brown, S. L. Schreiber, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 4947 – 4951; b) J. Choi, J. Chen, S. L. Schreiber, *Science* **1996**, *273*, 239 – 242; c) I. Remy, S. W. Michnick, *Nat. Methods* **2006**, *3*, 977 – 979.

[10] a) M. J. Robinson, M. H. Cobb, *Curr. Opin. Cell Biol.* **1997**, *9*, 180 – 186; b) B. J. Canagarajah, A. Khokhlatchev, M. H. Cobb, E. J. Goldsmith, *Cell* **1997**, *90*, 859 – 869.

[11] a) P. J. Roberts, C. J. Der, *Oncogene* **2007**, *26*, 3291 – 3310; b) K.-K. Wong, *Recent Pat. Anti-Cancer Drug Discovery* **2009**, *4*, 28 – 35.

[12] a) J. A. Smith, C. E. Poteet-Smith, Y. Xu, T. M. Errington, S. M. Hecht, D. A. Lannigan, *Cancer Res.* **2005**, *65*, 1027 – 1034, and references therein; b) C. Hecquet, G. Lefever, M. Valtink, K. Engelmann, F. Mascarelli, *Invest. Ophthalmol. Visual Sci.* **2002**, *43*, 3091 – 3098; c) T. L. Nguyen, *Anti-Cancer Agents Med. Chem.* **2008**, *8*, 710 – 716.

[13] C. Tong, H. Y. Fan, D. Y. Chen, X. F. Song, H. Schatten, Q. Y. Sun, *Cell Res.* **2003**, *13*, 375 – 383.

