Akt/protein kinase B (PKB) is a serine/threonine kinase that regulates a variety of cellular responses. To provide information on the spatial and temporal dynamics of Akt/PKB activity, we have developed genetically encoded fluorescent indicators for Akt/PKB. The indicators contain two green fluorescent protein mutants, an Akt/PKB substrate domain, flexible linker sequence, and phosphorylation recognition domain. A phosphorylation of the substrate domain in the indicators caused change in the emission ratio based on fluorescent resonance energy transfer between the two green fluorescent protein mutants. To let the fluorescent indicators behave as endothelial nitric-oxide synthase and Bad, which are endogenous Akt/PKB substrates, they were fused with the Golgi target domain and mitochondria targeting domain, respectively. The indicators phosphorylated with the endogenous substrates conferred their susceptibilities to phosphorylation by Akt/PKB. We showed that the Golgi-localized indicator responded to the stimulation with 17β-estradiol (E2) and insulin in endothelial cells. In addition, E2 elicited the phosphorylation of the mitochondria-localized indicator in the endothelial cells, but no phosphorylation was observed by E2 or by insulin of the diffusible indicator that has no target domain. The difference in the results with the three indicators suggests that the activated Akt/PKB is localized to subcellular compartments, including the Golgi apparatus and/or mitochondria, rather than diffusing in the cytosol, thereby efficiently phosphorylating its substrate proteins. E2 triggered the phosphorylation of the mitochondria-localized indicator, whereas insulin did not induce this phosphorylation, which suggests that the localization of the activated Akt/PKB to the mitochondria is directed differently between insulin and E2 via distinct mechanisms.

The Akt/protein kinase B was identified as a serine/threonine protein kinase with high homology with the protein kinases A and C. At the same time, this kinase was identified as the cellular homologue of the viral oncprotein v-Akt. Mammals have three closely related Akt isoforms, encoding Akt1, Akt2, and Akt3. All the three isoforms contain an N-terminal Pleckstrin homology domain, followed by a kinase domain and a C-terminal regulatory tail. Akt is an important regulator of various cellular processes including glucose metabolism, cell survival, and angiogenesis (1–3). Akt becomes activated by a wide variety of stimuli, including growth factors, cytokines, steroid hormones, and cellular stresses. Recent studies on the mechanism of Akt activation indicate that the phosphoinositide 3-kinase and its product phosphoinositide-3,4,5-triphosphate promote translocation of Akt to the plasma membrane and the phosphorylation at the two sites, Thr-308 and Ser-473, in Akt protein. The phosphorylation of the both sites is blocked by pretreatment of the cells with a phosphoinositide 3-kinase inhibitor, wortmannin. The activated Akt then phosphorylates substrates including glycogen synthase kinase-3, Bad, eNOS, caspase-9, and forkhead transcription factors (4–8). To achieve signaling specificity when the Akt signaling pathway is activated by stimuli that elicit different cellular responses, the exact location of the substrates in living cells may dictate which pathways are activated, but the precise mechanism remains poorly understood because of the lack of sufficient methods to study it.

Recently, we and other groups have reported genetically encoded fluorescent indicators for detecting protein phosphorylation in a single living cell (9–12). The approaches for detecting protein phosphorylation based on the fluorescent indicators have provided new information on the spatial and temporal regulation of activities of protein kinases and phosphatases.

Herein, we describe genetically encoded fluorescent indicators for Akt/PKB kinase activity, named Aktus (a fluorescent indicator for Akt phosphorylation that can be custom-made), eNOS-Aktus, and Bad-Aktus. eNOS-Aktus and Bad-Aktus are indicators for phosphorylation of eNOS and Bad, respectively. In addition, Aktus is a diffusible cytosolic indicator for phosphorylation by Akt. The common unit of the indicators is Aktus and is based on our general approach for visualizing protein phosphorylation in living cells (9), which contains two GFP mutants, an Akt substrate domain, a flexible linker sequence, and a phosphorylation recognition domain. We demonstrate that a FRET change between the two GFP mutants in the Aktus is induced by the phosphorylation of its Akt substrate domain. Almost all Akt substrates are known to localize to subcellular regions. eNOS is localized to the Golgi apparatus and cholesterol-rich microdomain of plasma membrane, caveolae in cells (13, 14), whereas Bad is present in mitochondria outer membrane (15, 16). By fusing the Aktus with the respectively subcellular localization domains for eNOS and Bad, the

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Kazuki Sasaki, Moritoshi Sato, and Yoshio Umezawa‡

From the Department of Chemistry, School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033 and Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan

‡ To whom correspondence should be addressed. Tel.: 81-3-5841-4351; Fax: 81-3-5841-8349; E-mail: umezawa@chem.s.u-tokyo.ac.jp.

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‡ To whom correspondence should be addressed. Tel.: 81-3-5841-4351; Fax: 81-3-5841-8349; E-mail: umezawa@chem.s.u-tokyo.ac.jp.

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* The abbreviations used are: eNOS, endothelial nitric-oxide synthase; FRET, fluorescence resonance energy transfer; E2, 17β-estradiol; TMRE, tetramethylrhodamine ethyl ester; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; CHO, Chinese hamster ovary; IR, insulin receptor; CPAE, calf pulmonary artery endothelial; ER, estrogen receptor.
Fluorescent Indicators for Akt/Protein Kinase B

Several fluorescent indicators for Akt were prepared as follows. CHO-IR-Akt cells expressing eNOS-Aktus were starved in 0.2% bovine serum albumin for 4–6 h and were then stimulated with 100 nM insulin for 15 min at 25 °C. After starvation in the steroid-free medium for 12 h, the CPAE cells infected with adenoviruses containing Akt and ER stimulated with 1 μM E2 for 20 min at 25 °C. The cells were lysed with an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After immunoprecipitation with the antibody for 1 h at 4 °C, the immunocomplex was precipitated using Protein G-Sepharose 4FF beads (Amersham Life Sciences). The sample was separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene fluoride membrane. The obtained signal was quantified using an image analyzer (LAS-1000plus; Fujifilm).

Immunofluorescence Microscopy—CPAE cells expressing eNOS-Aktus were fixed with 2% paraformaldehyde and were permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After 1 h of incubation with polyclonal rabbit anti-eNOS (C-20) antibody, the cells were washed with phosphate-buffered saline containing 0.2% fish skin gelatin and incubated with anti-rabbit IgG labeled with Cy5 for 30 min. The coverslips were mounted onto a slide and observed under a confocal laser-scanning microscope (LSM 510; Carl Zeiss).
AktusS136A (Fig. 2, cytosolic emission ratio in CHO-IR-Akt cells expressing domain. eNOS1 nase activity. Kz of fluorescent indicators for Akt kinase derived from Bad (RGRSRS*AP is the Akt substrate sequence) GFP with additional mutations (9). eNOS1 is the eNOS target domain (amino acids 1–35) and a spacer of 10 glycines. Tom20 is the mitochondria target domain derived from Tom20 (amino acids 1–33).

To examine the response of eNOS-Aktus to insulin, CPAE cells expressed with eNOS-Aktus were observed under a conventional fluorescence microscope, and the CFP/YFP emission ratio of the eNOS-Aktus in the Golgi apparatus was measured. Insulin stimulation triggered a rapid change in the emission ratio in CPAE cells infected with adenovirus-expressing Akt (Fig. 3B). Omission of the infection of Akt (Fig. 3B) abolished this FRET response to insulin, indicating that the response of eNOS-Aktus to insulin is caused by the Akt-dependent phosphorylation of eNOS-Aktus. To examine the response of the diffusible indicator Aktus to insulin, the cytosolic region of the CPAE cells transfected with Aktus was observed. Aktus exhibited no response to insulin in the CPAE cells infected with adenovirus containing Akt (Fig. 4). The different results between eNOS-Aktus and Aktus indicate that insulin-stimulated phosphorylation by Akt occurs in the Golgi apparatus but not in the cytosol. The increase in the efficiency of phosphorylation by Akt in the Golgi apparatus indicates that the activated Akt apparatus and caveolae (13, 14, 18). Both co-translational N-terminal myristoylation and post-translational palmitoylation (cysteines 15 and 26) of eNOS are required for the proper subcellular localization of eNOS (18). Previous work has shown that G2A eNOS is not myristoylated and does not localize to the Golgi apparatus or caveolae but is distributed throughout the cytosol (18, 34). Furthermore, upon stimulation with vascular endothelial growth factor, G2A eNOS was not phosphorylated at Ser-1179 by Akt (13) and did not produce nitric oxide (29). These results indicate that the localization of eNOS is critical for its phosphorylation and activation. To obtain spatial and temporal information on the dynamics of the phosphorylation of eNOS by Akt, we prepared eNOS-Aktus by fusing Aktus with the eNOS targeting domain. This eNOS-Aktus was in fact colocalized with endogenous eNOS in CPAE cells; the cells were transfected with eNOS-Aktus and immunolabeled with anti-eNOS antibody. Confocal fluorescence images illustrated that eNOS-Aktus was found to be colocalized with endogenous eNOS in CPAE cells (Fig. 3A). eNOS-Aktus and endogenous eNOS were found to localize in the Golgi apparatus (Fig. 3A, arrowhead), but eNOS-Aktus and endogenous eNOS in the caveolae were not found, which indicates that the majority of eNOS-Aktus and endogenous eNOS is localized in the Golgi apparatus in the CPAE cells.

Fluorescent Indicator for eNOS Phosphorylation—One of the major physiological roles of endothelial cells is to mediate the vasodilatory response to various agonists by NO production (31). Endothelial cells treated with insulin or estrogen have shown increased eNOS activity by Akt (4, 6, 32, 33). eNOS is located to specific intracellular domain, including the Golgi apparatus and caveolae (13, 14, 18). Both co-translational N-terminal myristoylation and post-translational palmitoylation (cysteines 15 and 26) of eNOS are required for the proper subcellular localization of eNOS (18). Previous work has shown that G2A eNOS is not myristoylated and does not localize to the Golgi apparatus or caveolae but is distributed throughout the cytosol (18, 34). Furthermore, upon stimulation with vascular endothelial growth factor, G2A eNOS was not phosphorylated at Ser-1179 by Akt (13) and did not produce nitric oxide (29). These results indicate that the localization of eNOS is critical for its phosphorylation and activation. To obtain spatial and temporal information on the dynamics of the phosphorylation of eNOS by Akt, we prepared eNOS-Aktus by fusing Aktus with the eNOS targeting domain. This eNOS-Aktus was in fact colocalized with endogenous eNOS in CPAE cells; the cells were transfected with eNOS-Aktus and immunolabeled with anti-eNOS antibody. Confocal fluorescence images illustrated that eNOS-Aktus was found to be colocalized with endogenous eNOS in CPAE cells (Fig. 3A). eNOS-Aktus and endogenous eNOS were found to localize in the Golgi apparatus (Fig. 3A, arrowhead), but eNOS-Aktus and endogenous eNOS in the caveolae were not found, which indicates that the majority of eNOS-Aktus and endogenous eNOS is localized in the Golgi apparatus in the CPAE cells.
results are the means ± S.D. of emission ratios from six different cells.

viruses expressing ER and Akt were transfected with eNOS-Aktus. E2 stimulation caused a significant decrease in the emission ratio of eNOS-Aktus in 3–8 min in the Golgi apparatus of the CPAE cells (Fig. 3B). When CPAE cells were expressed with either ER or Akt alone and stimulated with E2, respectively, no significant response of eNOS-Aktus was obtained (Fig. 3B), indicating that the response of eNOS-Aktus to E2 was caused by both ER- and Akt-dependent phosphorylation of eNOS-Aktus. On the other hand, Aktus under otherwise identical conditions exhibited no response to E2 (Fig. 4A). The different responses between eNOS-Aktus and Aktus suggest that Akt activated by E2 stimulation is localized to the Golgi apparatus.

To confirm that the responses of eNOS-Aktus to stimulation of insulin and of E2, respectively, reflect that eNOS in the Golgi apparatus is phosphorylated, immunoblotting analyses with anti-phospho-eNOS (Ser-1179) antibody were performed using the CPAE cells transfected with eNOS. The CPAE cells infected with Aktus exhibited insulin-dependent phosphorylation of eNOS (Fig. 3C, top). However, treatment with insulin of CPAE cells that were not infected with Akt did not induce the phosphorylation of eNOS (Fig. 3C, top). Moreover, the CPAE cells infected with both ER and Akt induced E2-dependent phosphorylation of eNOS, but the phosphorylation of eNOS upon stimulation with E2 was not detected in the CPAE cells infected with either ER or Akt alone (Fig. 3D, top). The results are consistent with the responses of eNOS-Aktus, indicating that the response of eNOS-Aktus represent the phosphorylation of eNOS. In addition, to confirm that the response of the cytosolic indicator Aktus reflects the phosphorylation of GSK3β, which is a substrate for Akt in the cytosol, immunoblotting analysis with anti-phospho-GSK3β antibody was performed using the CPAE cells transfected with GSK3β. Consistent with the response of Aktus, no phosphorylation of GSK3β was detected in the CPAE cells infected with Akt. The phosphorylation of GSK3β was detected in CHO-IR-Akt cells (Fig. 4B, top), where the response of Aktus was observed (Fig. 2D). The results indicate that the response of Aktus reflects the phosphorylation of the cytosolic protein GSK3β.

We have shown that the Akt activated by both E2 stimulation and insulin stimulation is localized to the Golgi apparatus, whereas E2 or insulin stimulation activates Akt in a different time-dependent manner. The different time dependence between stimulation with E2 and insulin is explained by the difference in the time dependence of phosphoinositide-3,4,5-triphosphate production, which precedes the Akt activation, as reported previously (6). It has been discovered that E2-induced activation of Akt occurs through a mechanism independent of insulin-induced activation of Akt (6).

Fluorescent Indicator for Bad Phosphorylation—Apoptosis is fundamental in the regulation of development and control of tissue homeostasis under conditions of cellular stress. Akt is known to exert antia apoptotic effects through several downstream targets. Among molecules central to the regulation of apoptosis in eukaryotes are members of the Bcl-2 family of proteins, including Bad, Bcl-2, and Bcl-XL. Bad is known to exert its apoptosis-promoting effects by heterodimerizing with Bcl-2 or Bcl-XL (36). Unphosphorylated Bad is capable of forming heterodimers with Bcl-2 or Bcl-XL localized to the mitochondrial outer membrane (15, 16). Datta et al. (17) showed that phosphorylation on Ser-136 of Bad by stimulation with
platelet-derived growth factor occurs via the phosphoinositide 3-kinase/Akt pathway in vitro and in vivo. Phosphorylated Bad is complexed with 14-3-3 protein and no longer interacts with Bcl-2 and Bcl-XL, allowing the inhibition of apoptosis (24).

To observe the phosphorylation of Bad in endothelial cells, Bad-Aktus, which is colocalized with endogenous Bad at the mitochondria outer membrane, is prepared by fusing Aktus with the mitochondrial targeting domain. To confirm that the Bad-Aktus in CPAE cells was localized in the mitochondria, CPAE cells transfected with Bad-Aktus were stained with TMRE, a mitochondrial marker. Confocal fluorescence images illustrated that the localization of Bad-Aktus found was the same as that of the mitochondria stained with TMRE (Fig. 5A). This merged image indicates that Bad-Aktus was localized in the mitochondria.

Estrogen is known to inhibit apoptosis of endothelial cells (37–39), but its mechanism of signal transduction pathways activated by estrogen is incompletely characterized. Although it was reported recently that E2 activates Akt in endothelial cells (6, 32, 35), it is not known whether stimulation with E2 induces Bad phosphorylation by Akt in endothelial cells. To examine whether Bad is phosphorylated by Akt upon stimulation with E2, CPAE cells infected with adenoviruses containing Akt and ER were transfected with Bad-Aktus. E2 was found to trigger a significant decrease in the CFP/YFP emission ratio of Bad-Aktus in 3–10 min in the mitochondria in CPAE cells (Fig. 5B). When CPAE cells were expressed with either ER or Akt alone, and stimulated with E2, respectively, no significant response with Bad-Aktus was observed (data not shown). This result clearly indicates that E2 stimulation induces the phosphorylation of endogenous Bad via an Akt- and ER-dependent pathway in the endothelial cells. The present result with Bad-Aktus suggests that E2-mediated inhibition of endothelial cell apoptosis may require the phosphorylation of Bad by Akt. Aktus exhibited no response to E2 in the cytosolic region of the CPAE infected adenovirus expressing ER and Akt (Fig. 4). The different results between Bad-Aktus and Aktus described above indicate that the E2-induced phosphorylation of Bad by Akt occurs in the mitochondria, which further suggests that the E2-activated Akt is also localized to the mitochondria.

In contrast to E2 stimulation, Bad-Aktus was not responsive to insulin stimulation in CPAE cells infected with adenoviruses expressing Akt (Fig. 5B). This result indicates that insulin stimulation induces no phosphorylation of endogenous Bad by Akt in endothelial cells. The results with Bad-Aktus suggest that the activated Akt that is induced by E2 is localized to the mitochondria.

**Fig. 3.** Cellular responses of eNOS-Aktus. A, subcellular localization of eNOS and eNOS-Aktus in CPAE cells. a, Cy5 immunolabeled with antibodies to eNOS. b, YFP in eNOS-Aktus. c, transmitted light view. d, the merged confocal image shows the colocalization of eNOS-Aktus with endogenous eNOS. After fixing and antibody staining as described under "Experimental Procedures," the cells were observed under a confocal laser-scanning microscope. B, time course of the emission ratio for eNOS-Aktus stimulated with E2 in CPAE cells infected with adenoviruses expressing ER and Akt (filled circles) and with either Akt (open circles) or ER (filled triangles). Time course of the emission ratio for eNOS-Aktus stimulated with insulin in the presence (filled squares) and absence (open circles) of adenovirus containing Akt. C, eNOS phosphorylation stimulated with insulin in CPAE cells. Immunoblot (IB) analysis was performed with anti-phospho-eNOS (Ser-1179) antibody (top) and with anti-eNOS antibody (bottom). D, eNOS phosphorylation stimulated with E2 in CPAE cells. Immunoblot analysis was performed with anti-phospho-eNOS (Ser-1179) antibody (top) and with anti-eNOS antibody (bottom).
mitochondria, but the activated Akt that is induced by insulin is not localized to the mitochondria; consequently, no phosphorylation of Bad takes place.

To confirm that the responses of Bad-Akt reflect the phosphorylation of Bad in the mitochondria, immunoblotting analyses with anti-Bad antibody were performed using the CPAE

**Fig. 4. Cellular responses of Aktus.** A, time course of the emission ratio for Aktus stimulated with E2 in CPAE cells infected with adenoviruses expressing ER and Akt (filled circles) and time course of emission ratio for Aktus stimulated with insulin in CPAE cells infected with adenovirus expressing Akt (open squares). B, GSK3β phosphorylation. Immunoblot (IB) analysis was performed with anti-phospho-GSK3β (Ser-9) antibody (upper) and with anti-GSK3β antibody (lower).

**Fig. 5. Cellular responses of Bad-Aktus.** A, subcellular localization of Bad-Aktus in CPAE cells. a, mitochondrial marker, TMRE. b, YFP in Bad-Aktus. c, transmitted light view. d, the merged confocal image of TMRE and Bad-Aktus was shown. B, the time course of the emission ratio for Bad-Aktus stimulated with E2 in CPAE cells infected with adenoviruses expressing ER and Akt (filled circles). The time course of Bad-Aktus stimulated with insulin in CPAE cells infected with adenovirus expressing Akt (open squares). C, Bad phosphorylation stimulated with E2 in CPAE cells. Mobility shift was examined by immunoblotting (IB) with anti-Bad antibody. D, Bad phosphorylation stimulated with insulin in CPAE cells. Mobility shift was examined by immunoblotting with anti-Bad antibody.
cells transfected with Bad. E2 stimulation of CPAE cells infected with both adenoviruses expressing ER and Akt caused a modification of Bad such that it migrated slower in SDS-polyacrylamide gels than Bad from unstimulated cells, indicative of E2-mediated phosphorylation of Bad (Fig. 5C). The phosphorylation of Bad upon stimulation with E2 was not detected in the CPAE cells infected with either ER or Akt alone (Fig. 5C). Insulin stimulation of CPAE cells infected with adenovirus expressing Akt was not able to induce the phosphorylation-dependent mobility shift of Bad (Fig. 5D). These results are in agreement with those of Bad-Akt, indicating that the response of Bad-Akt in fact represents the phosphorylation of Bad. The phosphorylation-dependent mobility shift of Bad can be regarded as the extent of the phosphorylation of Bad-Akt. Thus, by using the electrophoretic mobility shift assay of Bad, we examined what level of decrease in the CFP/YFP emission ratio is gained as a result of phosphorylation of the substrate domain in Bad-Akt. E2 stimulation of CPAE cells infected with both adenoviruses expressing ER and Akt induced an increase in phosphorylated Bad from 19% to 26% of the total Bad proteins (Fig. 5C). These values correspond to the emission ratio of Bad-Akt before and after E2 stimulation, respectively (Fig. 5B). The level of decrease in the CFP/YFP emission ratio of Bad-Akt was estimated to be $-6.5 \times 10^{-3}$ upon extrapolating the amount of the phosphorylated Bad with the mobility shift assay to 100% of the Bad proteins.

In conclusion, we have developed fluorescent indicators for the serine/threonine kinase Akt. We showed that eNOS-Aktus responded to the stimulation with E2 and insulin at the Golgi apparatus in the CPAE cells. In addition, E2 elicited the phosphorylation of Bad at the mitochondria in the CPAE cells. But no phosphorylation of Aktus was observed by E2 or by insulin. The difference in the results with the three indicators suggests that the activated Akt is localized to subcellular compartments, including the Golgi apparatus and/or mitochondria rather than diffusing in the cytosol, thereby efficiently phosphorylating its substrate proteins. E2 triggered the phosphorylation of Bad-Akt, whereas insulin did not induce the phosphorylation of Bad-Akt. The results suggest that the localization of the activated Akt is directed differently between stimuli, including insulin and E2, via distinct mechanisms to control phosphorylation of CPAE cells infected with only eNOS and Bad but also forkhead transcription factors and IκB kinases (4–8), both of which are localized in the nucleus. By replacing the targeting domains of the present indicators with those of forkhead transcription factors and IκB kinases, the genetically encoded indicators for forkhead transcription factors and for IκB kinases can also be prepared. Endogenous Akt isoforms are expressed at low levels in CPAE cells. Consequently, the endogenous Akt phosphorylated only a small portion of the expressed indicators. Overexpression of Akt was required to phosphorylate much of the remaining indicators, so that the activation of the Akt as a whole became detectable. Throughout this study, the overexpression was made with Akt1, which was the major isoform. Therefore, the conclusion was drawn basically with Akt1 that the activated Akt was localized in each particular subcellular compartment. Upon replacing Akt1 with Akt2 or Akt3 in the CPAE cells, possible distinctions if any between Akt1, -2, and -3 could be assessed. The present indicators and their applications are thus expected to contribute to the studies of a whole range of dynamics of the activated Akt in living cells.

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Kazuki Sasaki, Moritoshi Sato and Yoshio Umezawa

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