A Pair of Fluorescent Resonance Energy Transfer-based Probes for Tyrosine Phosphorylation of the CrkII Adaptor Protein in Vivo*\[S\]

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Adaptor proteins such as Grb2, CrkII, and Nck consist mostly of the Src homology (SH)\[^1\] and SH3 domains and play a pivotal role in the signal transduction of tyrosine kinases (1, 2). The SH3 domain binds constitutively to several cytoplasmic enzymes, whereas the SH2 domain binds to the target proteins that are phosphorylated on tyrosine. By having this property, adaptor proteins function to recruit SH3-bound cytoplasmic enzymes to the phosphotyrosine-containing proteins, which are mostly at the plasma membrane, in response to tyrosine kinase activation.

CrkII was originally identified as a human homolog of the Crk oncogene product of the CT10 retrovirus (3–5), and the homologs of CrkII have also been identified in Caenorhabditis elegans (6) and Drosophila melanogaster (7). All CrkII homologs consist of one SH2 and two SH3 domains. In v-Crk-transformed cells, two heavily phosphorylated proteins are bound to v-Crk (8), and they have been identified as p130\[^{Cas}\] (9) and paxillin (10). Because both p130\[^{Cas}\] and paxillin are phosphorylated by integrin stimulation (11, 12), and because CrkII expression increases cell migration (13), at least one function of CrkII seems to be involved in the regulation of cell adhesion and/or cellular movement. More recently, Wee1, which is known to inhibit cell cycle progression, has been shown to bind to the SH2 domain of CrkII and to accelerate apoptosis in a manner dependent on CrkII (14). These findings show that CrkII is involved in a variety of signaling cascades such as cell growth, migration, and apoptosis; however, it remains unknown how CrkII sorts out the signals to different downstream effectors.

Upon stimulation by a variety of growth factors, such as epidermal growth factor (EGF), nerve growth factor, insulin-like growth factor-I, and sphingosine 1-phosphate, CrkII is phosphorylated on Tyr\[^{221}\] (15–18). This phosphorylation induces intramolecular binding of the SH2 domain to Tyr\[^{221}\] (19–21), which may form a negative feedback loop or possess a positive role in signaling (22). Previously, we have developed an antiserum directed against oligo-peptides containing phosphorylated Tyr\[^{221}\] (15). This antibody enabled us to visualize growth factor-induced CrkII phosphorylation in fixed cells.

Recently, \[^{in vivo}\] probes based on fluorescent resonance energy transfer (FRET) technology have been developed for monitoring the intracellular signal transduction cascades (23). Here, we report on FRET-based \[^{in vivo}\] probes for CrkII phosphorylation by employing the property that CrkII changes its conformation by intramolecular binding of the SH2 domain to phosphorylated Tyr\[^{221}\]. Using of this probe, we visualize rapid and transient phosphorylation of CrkII in living cells.

EXPERIMENTAL PROCEDURES

Plasmids—pPicchu was derived from the pCAGGS eukaryotic expression vector (24) and encoded a chimeric protein, Picchu, which consisted of a yellow-emitting mutant of green fluorescent protein (YFP) (25), CrkII, and a cyan-emitting mutant of green fluorescent protein (CFP) from the amino terminus (see Fig. 1A). The carboxyl terminus of CrkII was truncated to the amino acids indicated as suffixes to the plasmid names. To construct the plasmids, we amplified each of the domains by polymerase chain reaction with primers con-
taining restriction enzyme recognition sites, cleaved them with restriction enzymes, and ligated them into the vector. Because pPicchu-236 yielded high FRET efficiency among the tested probes, the protein encoded by pPicchu-236 is called Picchu for simplicity in the text. Arg38, Tyr221, and Trp169 denote the amino acids 1–38, 221, and 169, respectively. The nucleotide sequences of the coding regions of pPicchu and pPicchu-X were deposited in the DDBJ/EMBL/GenBank.

### Results

**Phosphorylation Indicator of CrkII**—We designed a protein that consisted of CrkII and a pair of YFP and CFP, so that intramolecular binding of the SH2 domain of CrkII to phosphorylated Tyr221 brought CFP close to YFP and increased FRET from CFP to YFP (Fig. 1A). This recombinant protein was designated as a phosphorylation indicator of CrkII chimeric unit (Picchu). The prototype Picchu, however, showed only a marginal difference in the FRET efficiency between the phosphorylated and nonphosphorylated forms. Therefore, we truncated CrkII from the carboxyl terminus, expecting an increase in the FRET efficiency of the phosphorylated form. These truncated mutants of Picchu were co-expressed in 293T cells with or without c-Abl, and their emission profiles were examined at an excitation wavelength of 433 nm. FRET was most typically observed as an increase in an emission peak of 527 nm and a decrease in an emission peak of 475 nm. The emission ratio of 527/475 nm, which reflected the FRET efficiency, was increased in the presence of c-Abl (Fig. 1B). Among several mutants, Picchu-225, -230, and -236 yielded high FRET efficiency. We called Picchu-236 simply Picchu, and we used it in the following experiments.

To confirm that the increase in the emission ratio of 527/475 nm was caused by FRET, we digested Picchu with proteinase K. Because GFP and its mutants are resistant to proteinase K, this treatment will generate monomeric YFP and CFP. As shown in Fig. 1C, proteinase K treatment completely obliterated the emission peak at 527 nm, proving that the emission peak of 527 nm was caused by FRET (Fig. 1C).

Next, we confirmed by the use of two mutants, Picchu-R38V and Picchu-Y221F, that the increase in FRET was due to the intramolecular binding of the SH2 domain to the phosphorylated Tyr221. In Picchu-R38V, we replaced Arg38 with Val, which

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**Reagents and Methods**

- **293T** cells were transfected with pPicchu with or without pEBG-c-Abl. The cells were lysed and analyzed with fluorescence spectrometer at an excitation wavelength of 433 nm. FRET was most typically observed as an increase in a emission peak of 527 nm and a decrease in an emission peak of 475 nm. The emission ratio of 527/475 nm, which reflected the FRET efficiency, was increased in the presence of c-Abl (Fig. 1B). Among several mutants, Picchu-225, -230, and -236 yielded high FRET efficiency. We called Picchu-236 simply Picchu, and we used it in the following experiments.

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is an amino acid residue in the SH2 domain and is critical for the recognition of the phosphotyrosine. In Picchu-Y221F, Tyr221 was replaced with Phe. Picchu, Picchu-Y221F, and Picchu-R38V were expressed in 293T cells with or without c-Abl. We examined the FRET efficiency and the phosphorylation of these proteins (Fig. 1, D and E). c-Abl did not phosphorylate Picchu-Y221F or increase its FRET efficiency, which is expressed as the emission-intensity ratio of YFP to CFP. In contrast, c-Abl phosphorylated Picchu-R38V on tyrosine but did not increase its FRET efficiency. Thus, the intramolecular binding of the SH2 domain to the phosphorylated Tyr221 caused an increase in the FRET efficiency of Picchu.

**FRET Efficiency of Picchu in Living Cells**—Next, we examined the emission ratio of 530 ± 10 nm versus 475 ± 10 nm in living HT1080 cells expressing Picchu, Picchu-Y221F, and Picchu-R38V, with or without c-Abl. In the following figures, the cell images are presented in an intensity-modulated display mode, which associates color hue with emission ratio value and the intensity of each hue with the source image brightness. We found that Picchu was distributed evenly in the cells, including the nucleus. The distribution was very similar among Picchu, Picchu-Y221F, and Picchu-R38V (Fig. 2 A). As we observed in vitro, the FRET efficiency of Picchu, but not of Picchu-Y221F or Picchu-R38V, was increased in the presence of c-Abl. To confirm that the increase in the emission ratio was due to FRET, we photo-bleached YFP, which was expected to inactivate the photo-acceptor and to increase the emission from CFP. The result was exactly as we expected, confirming FRET from CFP to YFP (Fig. 2 B).

**Monitoring in Vivo of CrkII Phosphorylation by Picchu**—We next stimulated Picchu-expressing COS-1 cells with EGF, because its cognate receptor (EGFR) is known to phosphorylate CrkII on Tyr221 (15). Upon EGF stimulation, Picchu, but not Picchu-Y221F, was phosphorylated on tyrosine, concomitant with an increase in the FRET efficiency (Fig. 3 A). Of
The emission ratio of Picchu was increased only in the cytoplasm, but not in the nucleus, indicating that localization of phosphorylated Picchu was limited to the cytoplasm. To confirm that the increase in the emission ratio of Picchu was caused by FRET, we photo-bleached the acceptor, YFP, before EGF stimulation. As expected, only the nonphotobleached cells showed an increase in the emission ratio upon EGF stimulation (Fig. 3B).

Correlation of FRET Efficiency with Phosphorylation of Endogenous CrkII—We next examined whether the phosphorylation and FRET efficiency of Picchu correlated with the phosphorylation of the endogenous CrkII. Phosphorylation of both the endogenous CrkII and Picchu peaked at 10 min after EGF stimulation and returned to the basal level within 30 min in Picchu-expressing COS-1 cells (Fig. 4A). Then, in a similar condition, we monitored FRET efficiency by dual emission fluorescence microscopy (Fig. 4B and C, and supplementary material). The emission ratio of YFP to CFP started increasing within 1 min, reached to a plateau at 10 min, and returned to the basal level within 30 min. Thus, the emission ratio of YFP to CFP in Picchu-expressing cells faithfully reflected the phosphorylation status of the endogenous CrkII.

Requirement of Kinase Activity of EGFR for Increase in FRET Efficiency—We then confirmed the requirement of tyrosine kinase activity of EGFR by the use of a specific inhibitor, AG1478. As shown in Fig. 5A, AG1478, but not PP2, an inhibitor specific for Src family tyrosine kinases, inhibited the EGF-induced increase in FRET. We further examined EGF-induced CrkII phosphorylation in various cell types (Fig. 5B). Phosphorylation of CrkII, as judged by the increased emission ratio of Picchu, was observed in NIH3T3 cells, A431 epidermal carcinoma cells, and P13 cells upon EGF stimulation. P13 cells were mouse embryonic fibroblasts derived from a c- abl-deficient mouse; therefore, this observation showed that c-Abl was dispensable for EGF-dependent phosphorylation.

Increased Sensitivity and Spatio-temporal Information by Addition of CAAX Box—Under the conditions described above, we did not see any localized increase or decrease in the FRET efficiency within the cytoplasm. This appeared to be caused by rapid diffusion of Picchu, which was confirmed by fluorescence recovery after photobleaching. Therefore, to restrict the movement of the probe, we prepared Picchu-X, which contained the CAAX box of Ki-Ras at the carboxyl terminus of CFP. As shown

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in Fig. 6A, Picchu-X was localized mostly at the plasma membrane, whereas the prototype Picchu was localized diffusely in the cytoplasm. Owing to the reduction of the large pool of the nonphosphorylated form in the cytoplasm, Picchu-X showed significant improvement in sensitivity and time resolution. For the detection of EGF-induced phosphorylation of CrkII, Picchu required 10 ng/ml EGF, whereas less than 5 ng/ml was sufficient for Picchu-X (Fig. 6B). The EGF-induced increase in FRET efficiency reached a maximum within 2 min in Picchu-X-expressing cells, whereas it took about 10 min for Picchu-expressing cells to reach a plateau (Fig. 6C). Furthermore, this improvement in sensitivity and time resolution enabled us to observe where the CrkII phosphorylation was initiated. Within 40 s after the addition of EGF, the increase in the emission ratio started at the periphery and then moved toward the perinuclear region (Fig. 7 and supplementary material).

DISCUSSION

For delineating the signal transduction cascades in cells, it is essential to develop probes for spacio-temporal information on the activity of each signaling molecule. Antibodies specific to the phosphorylated form of signaling molecules such as anti-phospho-CrkII antibody are successfully used for such a purpose (15). FRET-based monitors are also used for detecting the activation of the signaling molecules. For example, CrkII labeled with fluorophore in vitro has been developed for detecting the conformational change in CrkII by phosphorylation (21). However, use of these probes in vivo is very limited because of the difficulty in their delivery into the cells. The development of a pair of GFP mutants that served as donor and acceptor overcame this problem and generated in vivo sensors for calcium (27), cAMP (28), and cGMP (29) or for monitoring the activities of protein kinase A (30), Rac (31), and Ras (32). After these successes, the development of Picchu showed that GFP-based FRET technology can be applied to the detection of the conformational change induced by tyrosine phosphorylation.

Previously, we reported that tyrosine-phosphorylated CrkII accumulated at the membrane ruffles upon EGF stimulation (15). However, in this study, we found that an increase in the FRET efficiency of Picchu occurs not only at the membrane ruffles but also in the cytoplasm. This apparent discrepancy may be explained as follows. Staining with anti-phospho-CrkII antibody reflects the net amount of phosphorylated CrkII, whereas the FRET efficiency of Picchu reflects the ratio of phosphorylated versus nonphosphorylated CrkII proteins. Thus, although CrkII is accumulated at the membrane ruffling sites, the ratio of phosphorylated versus nonphosphorylated CrkII may not differ significantly between the cytoplasm and the membrane ruffles. This interpretation agrees with our previous proposal that CrkII translocates to the plasma membrane upon EGF stimulation and passes from the plasma membrane to the cytoplasm upon phosphorylation by the EGF receptor (15).

A problem with probes using CFP and YFP as the FRET pair is the large leakage of donor emission in the acceptor channel at 535 nm (23). Hence, the excess of probes causes significant deterioration in detecting a signal-induced increase in FRET efficiency. Many SH2-containing signaling proteins, including CrkII, are localized mostly in the cytoplasm and translocate to the plasma membrane upon stimulation by growth factors (33). On these backgrounds, we could successfully improve Picchu in its sensitivity to EGF-induced phosphorylation by restricting its distribution to the plasma membrane. It should be kept in mind, however, that the CAA-fused Picchu reflects the phosphorylation status of CrkII at the plasma membrane. Therefore, authentic Picchu can be used for monitoring the level of net phosphorylation of CrkII, whereas CAA-fused Picchu should be used for examining the ongoing phosphorylation status of CrkII. Use of this pair of Picchu probes will shed light on the role of the CrkII adaptor protein in the physiologic signaling.
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