A Pair of FRET-based Probes for Tyrosine Phosphorylation of the CrkII Adaptor Protein in Vivo*

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RUNNING TITLE

In Vivo Probes for CrkII Phosphorylation
An adaptor protein, CrkII, which is involved in a variety of signaling cascades such as cell growth, migration, and apoptosis, becomes phosphorylated on Tyr\textsuperscript{221} upon stimulation. Here, we report on a FRET (fluorescent resonance energy transfer)-based sensor, which consists of CrkII sandwiched with cyan- and yellow-emitting variants of green fluorescent protein. This protein enabled us to monitor rapid and transient phosphorylation of CrkII upon epidermal growth factor (EGF) stimulation in a living cell. However, rapid diffusion of the probes prevented us from specifying where the phosphorylation started within the cell. To overcome this problem, we fused the CAAX box of Ki-Ras to the carboxyl terminus of this probe and restricted its localization mostly to the plasma membrane. With this modified probe, we found that EGF-induced phosphorylation of CrkII was initiated at the peripheral plasma membrane, moving toward the center of the cell. Moreover, this CAAX box-fused probe showed improvement in sensitivity and time resolution of the monitoring of CrkII phosphorylation. Thus, this pair of CrkII probes visualizes dynamic changes in the total and local levels of the tyrosine phosphorylation of CrkII in a living cell.
INTRODUCTION

Adaptor proteins such as Grb2, CrkII, and Nck consist mostly of the Src homology (SH) 2 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 C. elegans (6) and D. 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 p130Cas (9) and paxillin (10). Because both p130Cas 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 oligopeptides containing phosphorylated Tyr221 (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 of 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 Tyr221. By use 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 (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 PCR with primers containing 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 the simplicity in the text. Arg^{38}, Trp^{169}, and Tyr^{221} were replaced with Val, Leu, and Phe, in proteins encoded by pPicchu-R38V, -W169L, and -Y221F, respectively (15). A suffix, X, indicates that the CAAX box of Ki-Ras was fused to the carboxyl terminus of CFP, as described previously (26). Thus, from the amino terminus, Picchu-X consists of YFP (a.a. 1 to 239), spacer (Leu-Asp), CrkII (a.a. 1 to 236), spacer (Gly-Arg), CFP (a.a. 1 to 237), spacer (Gly-Arg-Ser-Arg), and CAAX box of Ki-Ras (a.a. 169 to 188). The nucleotide sequences of the coding regions of pPicchu and pPicchu-X were deposited in DDBJ/EMBL/GenBank. pEBG-cAbl was provided by B. J. Mayer at the University of Connecticut.

Cells and antibodies—COS-1, A431, NIH3T3, and HT1080 were purchased from the American Type Culture Collection or Japan Cell Resource Bank. An abl-deficient cell line, P13, was obtained from B. J. Mayer at the University of Connecticut. Anti-Crk monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-GFP rabbit serum and anti-phospho-CrkII antibody were developed in our laboratory (15).
Protein expression, in vitro spectroscopy, and immunoblotting—293T cells were transfected with pPicchu with or without pEBG-c-Abl. Thirty-six hours after transfection, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Triton X-100) and clarified by centrifugation. A fluorescence spectrum was obtained by use of an excitation wavelength of 433 nm with an FP-750 spectrofluorometer (JASCO Co, Tokyo). In another experiment, pPicchu, pPicchu-Y221F, and pPicchu-R38V with or without pEBG-cAbl were transfected into 293T cells grown on a pair of culture dishes. Cells on the first dish were used for in vitro spectroscopy analysis, and cells grown on the second dish were used for immunoblotting. Cells were lysed in SDS-sample buffer (40 mM Tris-HCl, pH 6.8, 0.1 M dithiothreitol, 1% SDS, 10% glycerol, and 0.05% bromophenol blue), boiled for 4 min, and clarified by centrifugation. Lysates from equal amounts of cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The filters were probed with the antibodies as described (15).

Imaging by dual-emission fluorescence microscopy—Cells were plated on a collagen-coated 35-mm-diameter glass-base dish (Asahi Techno Glass Co., Tokyo) and transfected with plasmids by use of either Superfect (Qiagen) or Lipofectamine 2000 (GIBCO-BRL). In some experiments, cells were serum-starved for 6 hrs and stimulated with 20 ng/ml epidermal growth factor (EGF). In another set of experiments, cells were pretreated with 10 nM AG1478 (Calbiochem), an inhibitor of EGF receptor tyrosine kinase, or 5 nM PP2 (Calbiochem), an inhibitor of Src tyrosine kinase, for 30 min. Cells were imaged on a Zeiss Axiovert microscope with a 75-W xenon arc lamp, two filter changers, a temperature-controlled CO2 chamber, and a cooled CCD camera, CoolSNAP-FX, controlled by MetaMorph software (Roper Scientific, Trenton, NJ) as described previously (27). For dual-emission ratio imaging of Picchu, we used a 12% neutral density filter,
an 86436 excitation filter (Chroma), a 455DRLP dichroic mirror (Omega), and two emission filters (Chroma), 86470 for CFP, 86535 for YFP, alternated by a filter changer. Cell images were analyzed with MetaMorph software. For the photobleaching of YFP, we illuminated a single cell by narrowing the field diaphragm, removed the neutral density filter, and photobleached with an 86500 excitation filter (Chroma) for 2 min. Cells were also imaged on an Olympus confocal laser microscope FV500ZX5 equipped with an Omnicrome HeCd laser.
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 non-phosphorylated 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 (23). 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 is an amino acid residue in the SH2
domain and is critical for the recognition of the phosphotyrosine. In Picchu-Y221F, Tyr\textsuperscript{221} 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.1D and 1E). 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 Tyr\textsuperscript{221} caused the 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, or 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. 2A). 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. 2B).

*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 Tyr\textsuperscript{221} (15). Upon EGF stimulation, Picchu, but not Picchu-Y221F, was phosphorylated on tyrosine, concomitant with an increase in the FRET efficiency (Fig. 3A). Of
note, 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 non-photobleached 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, 4C, and supplementary information for video). The emission ratio of YFP to CFP started increasing within 1 min, reached 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-\textit{abl}-deficient mouse; therefore, this observation showed that c-Abl was dispensable for EGF-dependent phosphorylation.

\textit{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\textsuperscript{2}. 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 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 non-phosphorylated 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 20 sec 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 information for video).
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 green fluorescent protein (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 A-kinase (30), Rac (31), and Ras (32). After these successes, the development of Picchu evidenced 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 non-phosphorylated CrkII proteins. Thus, although CrkII is accumulated at the membrane ruffling sites, the ratio of phosphorylated versus non-phosphorylated 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 CAAX-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 CAAX-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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FOOTNOTES

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The nucleotide sequences reported in this paper has been submitted to the DDBJ Data Bank with registration numbers 20010211140301.81036 and 20010511142937.77859.

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1The abbreviations used are: Src homology, SH; FRET, fluorescent resonance energy transfer; YFP, yellow-emitting mutant of green fluorescent protein; CFP, cyan-emitting mutant of green fluorescent protein; EGF, epidermal growth factor; GFP, of green fluorescent protein; IMD, intensity modulated display.

2K. Kurokawa and M. Matsuda, unpublished result.
FIGURE LEGENDS

FIG 1. Properties of Picchu. A, Schematic representation of phosphorylated and non-phosphorylated Picchu. YFP and CFP denote yellow-emitting mutant of GFP and cyan-emitting mutant of GFP, respectively. The sandwiched region consisting of SH2 and SH3 domains is from CrkII. Y and P denote Tyr221 and phosphate. B, Picchu probes with various carboxyl-terminal truncations were expressed with or without c-Abl in 293T cells. The number at the bottom indicates the last amino acid of each Picchu probe. Cells were lysed and analyzed with fluorescence spectrometer at an excitation wavelength of 433 nm. We used the emission intensities of CFP at 475 nm and YFP at 530 nm to calculate the emission ratio, YFP/CFP. C, Emission spectra of Picchu (excited at 433 nm) expressed with (red) or without c-Abl (blue) in 293T cells. One sample was treated with proteinase K (green) for 30 min before spectrum analysis. D and E, Emission ratio and immunoblotting analysis of Picchu-236 (WT), Picchu-236-Y221F mutant (Y221F), and Picchu-236-R38V mutant (R38V) expressed with or without c-Abl in 293T cells. Anti-phospho-Crk rabbit serum or anti-Crk monoclonal antibody was used for detecting Picchu.

FIG 2. Imaging of c-Abl-induced increase in the emission ratio of Picchu by dual-emission fluorescence microcopy. A, We used HT1080 cells expressing Picchu, Picchu-Y221F, or Picchu-R38V with or without c-Abl. Cells were placed in an inverted fluorescence microscope equipped with filter changers and a temperature-controlled CO2 chamber. CFP and YFP images were obtained with an 86436 excitation filter (Chroma), a 455DRLP dichroic mirror (Omega), and two emission filters (Chroma), 86470 for CFP, 86535 for YFP. The emission ratio YFP/CFP and the
intensity of CFP were used for imaging of Picchu-expressing cells in the IMD mode. Red and blue hues indicate emission ratios of 1.8 and 1.0, respectively. B, HT1080 cells expressing Picchu and c-Abl were prepared as in A. Pre-bleaching images of cells were obtained for 5 min. Then, only the cell indicated by a dotted yellow line was illuminated by narrowing the field diaphragm and photobleached at an excitation wavelength of 500 nm for 2 min, followed by imaging for 5 min. The time course of emission intensity of YFP and CFP of the photobleached cell is plotted in the right panel.

FIG 3. EGF-induced increase in the emission ratio of Picchu-expressing cells. A, Expression vectors encoding Picchu and Picchu-Y221F were transfected into COS-1 cells on three tissue culture plates each. Cells on one dish were stimulated with 20 ng/ml EGF for 10 min. Total cell lysates from unstimulated cells and from the EGF-stimulated cells were analyzed by immunoblotting with either anti-phospho-Crk antibody or anti-GFP antibody (upper panel). Cells on the last dishes were used for obtaining IMD images before and after EGF stimulation as in Fig. 2 (lower panel). B, COS-1 cells were transfected with the Picchu-encoding plasmid as in A. The cell indicated by a dotted line (top) was photobleached at an excitation wavelength of 500 nm for 2 min (center) and stimulated with EGF for 10 min (bottom).

FIG 4. Time course of CrkII phosphorylation and FRET efficiency. A, COS-1 cells expressing Picchu were stimulated with 20 ng/ml EGF as in Fig. 3. Cells were lysed at the indicated time points and analyzed by immunoblotting with anti-Crk antibody. Phosphorylated and non-phosphorylated CrkII are detected as slower- and faster-migrating forms of the CrkII protein. For detection of Picchu phosphorylation, Picchu was immunoprecipitated from the cell
lysates with anti-Crk monoclonal antibody and analyzed with immunoblotting with anti-phospho-Crk antibody. B and C, CFP and YFP images of a COS-1 cell expressing Picchu were obtained as in Fig. 2. The cell was stimulated with 20 ng/ml EGF. Time course of the emission ratio (YFP/CFP) is plotted in B. Representative IMD images are shown in panel C.

FIG 5. Monitoring of EGF-induced CrkII phosphorylation in the presence of tyrosine kinase inhibitors and in different cell lines. A, COS-1 cells expressing Picchu were left untreated or treated with AG1478 or PP2 as indicated and stimulated with 20 ng/ml EGF for 10 min. Cells were analyzed with either immunoblotting or dual-emission fluorescence microscopy as in Fig. 2. B, Cells indicated at the top were transfected with Picchu expression plasmid and stimulated with 20 ng/ml EGF for 10 min. IMD images were obtained as in Fig. 2.

FIG 6. Improved sensitivity and time resolution by the use of CAAX-box fused probe. A, Tangential CFP images of COS-1 cell expressing Picchu or Picchu-X were obtained with a confocal microscope with an 86470 emission filters (Chroma). B, COS-1 cells expressing Picchu or Picchu-X were stimulated by the indicated amount of EGF for 10 min. IMD images of pre- and post-EGF stimulation were obtained as in Fig. 2. C, COS-1 cells expressing Picchu or Picchu-X were analyzed by dual-emission fluorescence microscopy. Cells were stimulated with 20 ng/ml EGF and the emission ratio was monitored over time.

FIG 7. Improved space resolution by the use of CAAX-box fused probe. COS-1 cells expressing Picchu-X were serum-starved and analyzed by dual-emission fluorescence microscopy as in Fig. 2. Cell was stimulated with 10 ng/ml EGF and IMD images were obtained
every 20 seconds. Representative IMD images and a phase contract image of the pre-EGF stimulation are shown.
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