Mechanism of Csk-mediated Down-regulation of Src Family Tyrosine Kinases in Epidermal Growth Factor Signaling*\textsuperscript{\textregistered}

Hidetada Matsuoka, Shigeyuki Nada, and Masato Okada‡

From the Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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The Src family tyrosine kinases (SFKs) play pivotal roles as molecular switches that link a variety of extracellular cues to intracellular signaling pathway. The function of SFK is regulated by phosphorylation at the C-terminal regulatory site mediated by Csk. Recently a novel SFK target Cbp (or PAG) was identified as a membrane-anchored scaffold protein for Csk. To establish the mechanism of Csk/Cbp-mediated regulation of SFK in vivo, we observed dynamic changes in the interaction of Csk with Cbp by utilizing fusion proteins with modified green fluorescent proteins: cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Upon SFK activation induced by epidermal growth factor stimulation, fluorescent resonance energy transfer (FRET) response was detected transiently at membrane ruffles in COS1 cells co-expressing CFP-Csk and Cbp-YFP and in cells expressing a single-molecule FRET indicator consisting of CskSH2 and Cbp. Suppression of SFK by PP2 or use of a mutant Cbp that lacks the Csk binding site abolished the FRET response, although a dominant-negative form of Csk enhanced and sustained the FRET response, demonstrating that the FRET response is dependent upon the SFK activity. These observations show that Csk/Cbp-mediated down-regulation of SFK takes place at membrane ruffles in an early stage of epidermal growth factor signaling and suggest that the Csk/Cbp-based FRET indicators are useful for monitoring the status of SFK in living cells.

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† To whom correspondence should be addressed: Dept. of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-8297; Fax: 81-6-6879-8298; E-mail: okadam@iken.osaka-u.ac.jp.

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The abbreviations used are: SFK, Src family of tyrosine kinases; Csk, C-terminal Src kinase; Cbp, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; DIC, differential interference contrast.

The Src family of tyrosine kinases (SFK)\textsuperscript{1} are a family of nonreceptor protein tyrosine kinases that associate with the inner surface of the plasma membrane by means of a fatty-acylated amino terminus (1). SFKs serve as molecular switches that regulate a variety of cellular events including cell growth, division, and differentiation (2). SFKs are strictly regulated by the phosphorylation of two tyrosine residues: autophosphorylation of a tyrosine in the kinase domain is required for full activity, whereas phosphorylation of a tyrosine in the C-terminal region abolishes activity (3). There is ordinarily an equilibrium between an inactive state in which the phosphorylated C-terminal tyrosine binds intramolecularly to the SH2 domain, and a “primed” state in which the C-terminal tyrosine is dephosphorylated. In response to an extracellular signal, the primed SFK can be activated by accumulation of SFK itself or of other components that bind to its SH2 or SH3 domains. Thus, it is thought that SFK activity is controlled in part by the relative activities of a kinase and a phosphatase that target the C-terminal tyrosine.

Phosphorylation of the C-terminal tyrosine is dependent upon another PTK, C-terminal Src kinase (Csk) (4, 5). The importance of Csk as a negative regulator of SFK and the absence of any functional redundancy have been demonstrated by the observation that Csk knockout mice that die before birth exhibit constitutive activation of many SFKs. Csk is a cytoplasmic protein tyrosine kinase consisting of an SH3, an SH2, and a kinase domain (4). There is evidence that the SH3 and SH2 domains of Csk are essential for the regulation of SFK, and that Csk can be recruited to the membrane when SFKs are in an active state (6, 7), although the molecular mechanism underlying these events remains unknown. Recently, we and others (8, 9) have identified a transmembrane phosphoprotein, known as Csk-binding protein (Cbp) or phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), which binds tightly to the SH2 domain of Csk. Cbp is located exclusively in so-called lipid rafts in which cholesterol, glycosphingolipid, and a variety of membrane-anchored signaling molecules including SFK accumulate. Lipid rafts are thus viewed as platforms for signaling across the membrane. The interaction of Cbp with Csk is also dependent upon the phosphorylation of its Tyr-314 by SFK (8–10). Based on these lines of evidence, a model of feedback regulation of SFK has been presented according to which activation of SFK by an extracellular stimulus leads to the phosphorylation of Cbp; this results in recruitment of Csk to lipid rafts and is followed by termination of SFK function (11–13). However, there is no conclusive evidence that this actually occurs in living cells.

Recently, in vivo indicators based on fluorescence resonance energy transfer (FRET) technology have been developed for monitoring intracellular signal transduction cascades (14–17). FRET is the nonradioactive transfer of excited-state energy between two fluorophores in FRET. With the development of spectrally different fluorophores and improved technologies for fluorescence measurements, fluorescent indicators have permitted visual-
FIG. 1. Co-localization of Cbp and Csk at membrane ruffles. A, time-lapse imaging of Cbp and Csk. COS1 cells were co-transfected with Cbp-YFP and CFP-Csk for 12 h, serum starved for 12 h, and stimulated with EGF. Time-lapse images were obtained using YFP and CFP every 10 s. Images of Cbp-YFP (red) and CFP-Csk (green) are shown at the indicated time points. Merged images are shown in the bottom panels (video images are available in Supplemental Material 1). B, Cbp-YFP and CFP-Csk co-expressing cells were treated with PP2 for 30 min and stimulated with EGF. Images of CFP-Csk obtained at 0 and 10 min are shown. C, CbpF314-YFP and CFP-Csk co-expressing cells were stimulated with EGF. Images of CFP-Csk obtained at 0 and 10 min are shown.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal anti-Cbp antibodies were raised against a peptide corresponding to 155 amino acids (residues 53–208) of Cbp and affinity-purified with peptide-coupled resin. Anti-GFP polyclonal antibody, anti-Fyn polyclonal antibody (FYNS1), anti-Src monoclonal antibody (Ab-1), anti-phosphotyrosine ERK antibody, and anti-phosphotyrosine monoclonal antibody (anti-pY, 4G10) were purchased from Molecular Probes, Santa Cruz Biotechnology, Oncogene Research, Cell Signaling Technology, and Upstate Biotechnology, respectively. Anti-Src polyclonal pTyr418 and anti-Src polyclonal pTyr529 were purchased from BioSource. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Westlon Laboratories Inc. The Fyn expression vectors, pME18Sneo-Fyn and pME18Sneo-SECFP, were obtained from T. Yamamoto (University of Tokyo). Zymed Laboratories Inc. provided the following Fyn expression vectors: pcDNA3-SE10C and pRSET-SECFP. PP2 and EGF were obtained from Calbiochem. Rabbit polyclonal anti-Cbp antibodies were provided by A. Miyawaki of the Brain Science Institute, RIKEN, Wako-shi, Japan. The yfp and cfp genes were amplified from these vectors by PCR and ligated into vectors pEGFP-N1 and pEGFP-C1 (Clontech) using the KpnI and NotI restriction sites (for the N1 vector) and NheI and SalI restriction sites (for the C1 vector), respectively. Anti-Src polyclonal pTyr418 and anti-Src polyclonal pTyr529 were purchased from BioSource. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Westlon Laboratories Inc. The Fyn expression vectors, pME18Sneo-Fyn and pME18Sneo-SECFP, were obtained from T. Yamamoto (University of Tokyo).

Immunoblot analysis of the interaction between Cbp and Csk. A, COS1 cells were co-transfected with Cbp-YFP and CFP-Csk. After stimulation with EGF for the indicated times, lysates were prepared and analyzed by immunoprecipitation and immunoblot assays to detect the activations of SFK and Erk1/2, phosphorylation of Cbp, and interaction of Cbp-YFP with CFP-Csk. COS1 cells co-expressing Cbp-YFP and CFP-Csk were incubated with or without PP2 for 30 min and stimulated with EGF for 10 min. The biochemical events were detected as in A. C, COS1 cells co-expressing CbpF314-YFP and CFP-Csk were stimulated with or without EGF for 10 min. The biochemical events were detected as in A.
Fig. 3. Analysis of in vitro FRET between Cbp-YFP and CFP-Csk. A, COS1 cells were co-transfected with Cbp-YFP and CFP-Csk with or without Fyn or FynF528, and their cleared lysates were prepared (left panel). Cleared lysates of COS1 cells co-expressing CbpF314-YFP and CFP-Csk were also prepared (right panel). The expression of fusion proteins and Fyn were detected by immunoblot assay, and the interaction of Cbp with Csk and the phosphorylation of Cbp were analyzed by immunoprecipitation assays with anti-Cbp antibody followed by immunoblotting with anti-GFP or anti-pY antibody. B, fluorescence spectra were analyzed with a fluorescence spectrometer at an excitation wavelength of 433 nm. The individual fluorescence spectra of total cell lysates prepared from cells expressing the indicated genes are shown. C, emission ratios (YFP/CFP) were calculated from the emission intensities of CFP and YFP at 475 nm and 527 nm, respectively.

c-transfected with combinations of expression vectors, as shown in each figure legend. The transfected cells were seeded onto 100-mm dishes, serum-starved for 12 h, and stimulated with 100 ng/ml EGF. After incubation for various times, samples were washed with ice-cold phosphate-buffered saline and lysed with ice-cold ODG buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM Na3VO4, 20 mM NaF, 10 μg/ml leupeptin, 1 μg phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation at 12,000 × g for 30 min at 4 °C. In other experiments, the transfected cells were treated with the SFK inhibitor, PP2 (2 μM), for 30 min before EGF stimulation. The cells were lysed in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromphenol blue); equal volumes were fractionated by SDS-PAGE and transferred to nitrocellulose transfer membranes (Schleicher & Schuell). The membranes were blocked with T-TBS or T-TBS containing 1% bovine serum albumin, incubated with primary antibodies for 3 h, washed with T-TBS, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, and again washed with T-TBS. Immunoreactivity was visualized with a chemiluminescence system (PerkinElmer Life Sciences). For the immunoprecipitation assay, cell lysates were incubated with anti-GFP or anti-Cbp antibody coupled with protein G-Sepharose (Amersham Biosciences) at 4 °C for 3 h. The washed beads were subjected to immunoblot analysis with anti-phosphotyrosine (4G10), anti-GFP, or anti-Csk antibodies.

In Vitro Spectrophotometric Measurements—COS1 cells expressing Cbp-YFP and CFP-Csk or Chimera with or without pME18Sneo-Fyn and pME18Sneo-FynF528 were lysed in ODG buffer and cleared as described above. Lysates were also prepared from COS1 cells co-expressing CbpF314-YFP and CFP-Csk and those expressing F314Chimera. Expression of individual proteins was confirmed by immunoblotting with anti-GFP, anti-Cbp, and anti-Fyn antibody. A fluorescence spectrum was obtained at an excitation wavelength of 433 nm with an F-2500 fluorescence spectrophotometer (Hitachi Co, Tokyo, Japan). To confirm FRET, cell lysates were incubated with 200 μg/ml proteinase K or 2 μM PP2 at 37 °C for 30 min, and cleared supernatants were analyzed by immunoblotting and spectrophotometric assay.

Imaging of SFK Activation—To monitor the activation of SFK in the living cell, COS1 cells were transfected with plasmids Cbp-YFP/CFP-Csk, CbpF314-YFP/CFP-Csk, Cbp-YFP/CFP-CskR222, Chimera, and F314Chimera. Twelve hours later, they were plated onto a 35-mm-diameter collagen-coated glass-based dish (Asahi Techno Glass Co., Tokyo, Japan), serum-starved for 12 h, and stimulated with 100 ng/ml EGF with or without pre-treatment with 2 μM PP2 for 30 min. After EGF stimulation, the cells were imaged every 10 s with an Olympus IX71 microscope equipped with a 75-W xenon arc lamp, two filter changers, a temperature-controlled chamber, and a cooled charge-coupled device camera, CoolSNAP HQ (Roper Scientific, Trenton, NJ), controlled by MetaMorph software (Universal Imaging, West Chester, PA). For dual-emission ratio imaging of FRET, we used the 86006 filter set (Chroma), an S436/10 and S492/18 excitation filter, a 455DRLP dichroic mirror (Omega), and two emission filters, S465/30m for CFP and S535/30m for YFP, alternated by a filter changer. The cells were illuminated with a 75-W xenon arc lamp through a 10% neutral density filter (Omega Optical Inc.) and a 60% oil immersion objective lens. The ratio image of YFP/CFP was created with MetaMorph software and used to represent FRET efficiency. For photobleaching YFP, cells were illuminated without a neutral density filter and photobleached with an S492/18 excitation filter for 1 min.

RESULTS

Co-localization of Csk with Cbp in EGF-stimulated COS1 Cells—To monitor the changes in localization of Cbp and Csk in
living cells, Cbp-YFP and CFP-Csk were expressed in COS1 cells, and their localization was observed by fluorescence microscopy. We confirmed that these tagged proteins behaved just like the endogenous proteins by immunocytochemical analysis (data not shown). Cells co-expressing Cbp-YFP and CFP-Csk were stimulated with EGF, and images of CFP and YFP, along with differential interference contrast (DIC) images, were obtained every 10 s by time-lapse microscopy. CFP-Csk was present in the cytoplasm of resting cells and translocated to the plasma membrane and membrane ruffles after EGF stimulation (Fig. 1A, top). Furthermore, CFP-Csk was strictly co-localized with Cbp-YFP at the membrane ruffles (Fig. 1A, bottom). CFP-Csk was not translocated when cells were pre-treated with the SFK inhibitor, PP2 (Fig. 1B), or when it was co-expressed with a mutant form of Cbp, Cbp F314-YFP, that lacked the binding site Tyr-314 (Fig. 1C). These observations suggest that Csk associates with Cbp when phosphorylated by SFK.

**Direct Interaction between Csk and Cbp in Cell Lysates**—To verify the Csk-Cbp interaction in vitro, COS1 cells expressing Cbp-YFP and CFP-Csk were stimulated with EGF, and lysates were subjected to biochemical analyses (Fig. 2A). After EGF stimulation, tyrosine phosphorylation of cellular proteins including the EGF receptor and Erk1/2 increased, and activation of SFK, as indicated by phosphorylation of its autophosphorylation site, Tyr-418, peaked at about 10 min after stimulation. This peak was accompanied by an increase in tyrosine phosphorylation of Cbp and in the level of co-immunoprecipitated CFP-Csk with Cbp-YFP, although there was significant basal interaction because of basal phosphorylation of Cbp. The involvement of SFK is supported by the observation that pretreatment with PP2 suppressed these effects, although activation of Erk1/2 persisted, because the Erk1/2 pathway is independent of SFK signaling (Fig. 2B). In COS1 cells expressing Cbp F314-YFP instead of Cbp-YFP, co-immunoprecipitation of Cbp F314-YFP with CFP-Csk was not detected in either the resting or activated state (Fig. 2C). It is clear, therefore, that Csk and Cbp interact when Cbp is phosphorylated by activated SFK.

**Detection of Interaction between Csk and Cbp by FRET in Vitro**—We examined the interaction between CFP-Csk and Cbp-YFP by FRET to confirm the interaction between Csk and Cbp and to see if this interaction could be useful for monitoring the activation of SFK in living cells. To evaluate the FRET efficiency of these indicators, we first carried out in vitro spectrometric studies. The indicators were co-expressed in COS1 cells with Fyn, a member of the SFK family, or a constitutively active form of Fyn, Fyn F528, to induce efficient phosphorylation of Cbp. Cell lysates were prepared, and expression of each of the proteins was confirmed (Fig. 3A). Tyrosine phosphorylation of Cbp-YFP proved to be dependent upon the expression of Fyn or Fyn F528, whereas co-precipitation of CFP-Csk was dependent upon the phosphorylation of Tyr-314 (Fig. 3A). The fluorescence emission profiles of these lysates were obtained at an excitation wavelength of 433 nm (Fig. 3B). FRET is typically observed as an increase in an emission peak at 527 nm and a decrease in an emission peak at 475 nm (14), and the ratio of emission at 527 nm and 475 nm is used to measure FRET. As shown in Fig. 3C, the emission ratio of Cbp-YFP/CFP-Csk increased in the presence of Fyn or Fyn F528. When Cbp F314-YFP replaced Cbp-YFP, no change in FRET was observed even in the presence of Fyn. To confirm that the increase in the emission ratio was caused by FRET, the cell lysate was treated with proteinase K. Because GFP and its derivatives are resistant to proteinase K (14), this treatment removed the protein tags and, as shown in Fig. 3B, greatly diminished the emission peak at 527 nm.

**Interaction of Csk with Cbp in the Living Cell**—Next we examined time-lapse images of the interaction of Csk with Cbp in the living cell. COS1 cells co-expressing Cbp-YFP and CFP-Csk were stimulated with EGF, and images of CFP, YFP, and DIC were obtained every 10 s. YFP/CFP ratio images were used to measure FRET in the intensity-modulated display mode. The mosaic of ratio images is presented using eight colors from blue to red, corresponding to low and high ratios, respectively. A change in the YFP/CFP emission ratio was detected along membrane ruffles upon EGF stimulation (Fig. 4A). The maxi-
mum signal was detected 5–10 min after stimulation, which is in good agreement with the activation of SFK by EGF stimulation. No increase in the YFP/CFP emission ratio was observed when the cells were pre-treated with PP2 (Fig. 4 B) or when CbpF314-YFP was used (Fig. 4 C). In addition, photobleaching of YFP completely removed the YFP signal but increased the emission of CFP at membrane ruffles (Refs. 18, 21; data not shown). FRET signals, therefore, reveal that CFP-Csk and Cbp-YFP interact at membrane ruffles.

Requirement of Csk for SFK Regulation in Living Cells—To examine the role of Csk in the feedback regulation of SFK, we employed a kinase-deficient mutant of Csk. Over-expression of a kinase-deficient Csk leads to activation of SFK (22–24). To confirm this in our system, CskR222 with Arg replacing Lys-222 was tagged by CFP and co-expressed in COS1 cells with Cbp-YFP. As shown in Fig. 5 A, the level of CFP-CskR222 in cell lysates and the amount co-immunoprecipitated with Cbp-YFP was greater than with endogenous Csk. Moreover, activation of SFK and tyrosine phosphorylation of Cbp was detected even without EGF stimulation and was sustained at a high level for up to 30 min after EGF stimulation. These results reveal that CFP-CskR222 acts in a dominant-negative fashion to induce the constitutive activation of SFK. We then analyzed YFP/CFP ratio images for CFP-CskR222/Cbp-YFP in living COS1 cells. When the cells were stimulated with EGF, an increased YFP/CFP emission ratio was detected at membrane ruffles even before EGF stimulation, and the effect intensified after EGF stimulation (Fig. 5 B). It was sustained for 30 min, which is consistent with the prolonged activation of SFK. Photobleaching of YFP increased the CFP signals (data not shown). These in vitro and in vivo observations show that Csk is required at the membrane ruffles to shut off the transiently activated SFK, supporting the presence of negative feedback regulation of SFK during EGF signaling.

Monitoring of SFK Activation with a Single-molecule FRET Indicator—The FRET signals obtained with the combination of CFP-Csk and Cbp-YFP provided evidence that EGF-induced activation of SFK, and Csk-mediated regulatory events, occur
Because activation of SFK always accompanies phosphorylation of Cbp, it seemed possible that FRET signals that depended upon phosphorylation of Cbp would be useful for monitoring the activity status of SFK without disturbing its function. We therefore constructed a single-molecule FRET probe by utilizing regions of Cbp containing membrane-localization signals and phosphorylation sites and the SH2 domain of Csk (Fig. 6A). We expected that, with this indicator (Chimera), the interaction between the Cbp substrate region and the SH2 domain of Csk would enhance FRET from the C terminus of CFP to the N terminus of YFP in a phosphorylation-dependent manner.

In vitro spectrophotometric measurements revealed that the emission ratio for Chimera did increase in COS1 cells co-expressing Fyn or FynF528, whereas there was no FRET change when an F314Chimera lacking the Csk binding site was used (Fig. 6C and D). Treatment with PP2 also removed the emission peak of Chimera at 527 nm (Fig. 6C and D). Immunoprecipitation and immunoblot analyses of these cell lysates showed that tyrosine phosphorylation of Chimera was dependent upon the expression of Fyn or FynF528 (Fig. 6B), and that PP2-treatment suppressed SFK activity as well as phosphorylation of Chimera (Fig. 6B). These results demonstrate that the increase in the emission ratio of Chimera was caused by FRET, and that the FRET response was dependent upon the activity of SFK.

The correlation between the activation of endogenous SFK and tyrosine phosphorylation of Chimera was further confirmed in cells stimulated with EGF. As shown in Fig. 7A, tyrosine phosphorylation of Chimera peaked 10 min after EGF stimulation in parallel with activation of SFK. In addition, pretreatment with PP2 suppressed both activation of SFK and tyrosine phosphorylation of Chimera (Fig. 7A, middle panel). In cells expressing F314Chimera, EGF stimulation activated SFK, whereas tyrosine phosphorylation of F314Chimera was barely detected (Fig. 7A, right panel).

We then attempted to obtain time-lapse images of SFK activation in living COS1 cells using Chimera. The cells were stimulated with EGF, and images of the YFP/CFP emission ratio were generated. An increase in the YFP/CFP emission ratio was observed at membrane ruffles upon EGF stimulation.
FRET began to increase within 1 min, reached a plateau at about 10 min, and then returned to baseline. Photobleaching of YFP induced an increase in CFP emission (14, 17), indicating that the increase in YFP/CFP emission ratio was caused by FRET (data not shown). No increase in YFP/CFP emission ratio occurred upon EGF stimulation with the F314Chimera or after treatment with PP2 (Fig. 7, C and D). These results demonstrate that the YFP/CFP emission ratio of Chimera reflects the activation of endogenous SFK at membrane ruffles.

**DISCUSSION**

GFP-based FRET technology is increasingly being used to visualize intracellular signaling in living cells (21, 25–30). With this technique we have examined the interaction between Csk (a negative regulatory kinase for SFK) and Cbp (a transmembrane protein that can recruit Csk when phosphorylated by SFK). We have also analyzed when and where SFK is activated and regulated in living cells. The results reported above clearly show that the observed FRET signals represent the direct interaction of Csk and Cbp in living cells. It was shown previously through biochemical analyses that Cbp phosphorylated by SFK is involved in the membrane localization of Csk and the Csk-mediated down-regulation of SFKs (8, 9). However, there has been no direct evidence for the presence of such a regulatory system in living cells. In this study, we showed that the active Csk was indeed recruited to Cbp when phosphorylated...
by SFK; its rapid dissociation from the complex was followed by a loss of SFK activity. These ordered reactions support the occurrence of negative feedback regulation of SFK in living cells. To further verify the contribution of Csk, our FRET analysis showed that Csk activity is required for the feedback regulation of SFK that leads to prompt termination of SFK signaling.

The use of two FRET indicators showed that Csk is transiently recruited to membrane ruffles in which Cbp is phosphorylated by SFK upon EGF stimulation. When activated by EGF, the EGF receptor accumulates in lipid rafts that are harbored in membrane ruffles (31, 32) and associates with and activates SFK present in the rafts. Cbp, co-localized with SFK in lipid rafts, is also enriched in membrane ruffles upon EGF stimulation, and its phosphorylation status is consistent with the activity status of SFK. Furthermore, in vitro studies show that Cbp is an excellent substrate for SFK (33). Taken together, these observations suggest that the phosphorylation of Cbp is a hallmark of SFK activation, and that the Cbp-Csk interaction represents, albeit indirectly, the activity status of SFK at the exact locations where SFK signaling is taking place. These considerations led us to expect that the FRET indicators could be used to monitor the activity of SFK in vitro. However, because the observations with the kinase-defective Csk showed that the two molecular indicators are functional and sometimes affect cellular events, we constructed a single-molecule FRET probe using the interaction between the phosphorylated Tyr-314 of Cbp and the SH2 domain of Csk. The FRET signal from this indicator accurately reflected the activation of SFK in vitro, and the time-lapse images of its FRET signals were almost equivalent to those obtained with the Cbp-YFP/CFP-Csk indicators. This result demonstrates that this single-molecule indicator can detect the status of SFK in living cells. We are now extending the use of this indicator for detecting other SFK signaling events in lipid rafts, such as cell adhesion signaling mediated by integrins.

Src can physically associate with the EGF receptor when activated by EGF stimulation (34–36). However, the nature of this interaction and the physiological role of Src activation in EGF signaling remain unclear. Recently, mechanisms by which SFK may affect ligand-induced endocytosis of the EGF receptor and Cbp in lipid rafts are described (37). This new model predicts that SFK can reside in lipid rafts and can be recruited to membrane ruffles upon EGF stimulation, as well as during dynamin-mediated vesicle budding. These observations suggest that SFK activation is required for the initial step of receptor endocytosis. We are now seeking the SFK target that triggers receptor endocytosis.

In this study, we showed that Csk was specifically recruited to phosphorylated Cbp upon EGF stimulation. However, it was previously reported that other SFK targets such as paxillin and FAK could recruit Csk to a location where they were phosphorylated by SFK (6). Thus it may be that multiple and/or complementary negative feedback loops regulate SFK. To separate these multiple systems and evaluate the role of each, more specific indicators containing a specific localization signal and a stable Csk binding site should be designed. In this study, we developed a single indicator to visualize the activity status of SFK. Because Cbp is exclusively localized at lipid rafts, this indicator should be useful for monitoring SFK signaling originating from lipid rafts, such as EGF and T-cell receptors, and cell adhesion signaling. The progressive presence of human cancers associated with the elevated expression and activity of SFK (41–43), and it has been suggested that the metastatic activity of cancer cells in particular can be defined by the status of their SFK activity (24). If an indicator that precisely reflected this status could be designed, it would be very useful for screening drugs that decrease the deregulated activity of SFK. Meanwhile, the substrate-based indicator for SFK activity used above should be useful for investigating the regulation of SFK in a variety of cellular processes, although further improvements may be necessary.

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