

The amino-terminal B-Raf-specific region mediates calcium-dependent homo- and hetero-dimerization of Raf

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B-Raf is a key regulatory molecule of the mitogen-activated protein kinase kinase (MEK). B-Raf differs from the other Raf isoforms in that it has a long amino-terminal region. By the use of probes based on the principle of fluorescence resonance energy transfer, we found that this amino-terminal B-Raf-specific region is essential for homo-dimerization of B-Raf and hetero-dimerization of B-Raf and c-Raf at the plasma membrane, followed by phosphorylation of Thr¹¹⁸ in the amino-terminal B-Raf-specific region. HeLa cells expressing B-Raf, but not c-Raf, or a B-Raf mutant lacking the B-Raf-specific region, showed enhanced MEK phosphorylation upon stimulation with a calcium agonist. Furthermore, increases in the intracellular calcium concentration were found to be necessary for dimerization and sufficient for the plasma membrane translocation of B-Raf. Notably, in calcium ionophore-stimulated HeLa cells, B-Raf could propagate signals to MEK under the basal level of GTP-Ras. Thus, we propose that the hitherto unidentified function of the B-Raf amino-terminal region is to mediate calcium-dependent activation of B-Raf and the following MEK activation, which may occur in the absence of Ras activation.

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Introduction

Three Raf proteins, A-Raf, B-Raf, and c-Raf, together play a pivotal role in transducing growth signals from Ras to the mitogen-activated protein (MAP) kinase kinase (MEK) (Avruch *et al.*, 1994; Daum *et al.*, 1994). In addition to Ras, several kinases and scaffold proteins regulate Raf proteins either positively or negatively (reviewed in Kolch, 2000; Morrison and Davis, 2003). Although there are cell type- and isoform-specific regulatory mechanisms, all three isoforms seem to be activated by a common scheme (reviewed in Wellbrock *et al.*, 2004). Raf proteins adopt closed inactive conformation by means of a 14-3-3 dimer, which intramole-

cularly crosslinks the amino and carboxyl sides of the catalytic domains. Upon Ras activation, Raf proteins translocate to the plasma membrane, bind to Ras-GTP, and adopt an open conformation (Terai and Matsuda, 2005). Subsequent phosphorylation of the catalytic region and/or negative charge regulatory region (N-region) renders Raf proteins catalytically active.

Unlike the other Raf genes, the B-Raf gene has a high incidence of activating mutations among various types of human malignant tumors. This implies that the regulation of B-Raf may differ from that of the other two isoforms. Structurally, B-Raf differs from the other two in that B-Raf has a long amino-terminal region before conserved region 1, and in that the N-region is constitutively charged negatively. The latter property partly explains why B-Raf possesses high basal activity and is liable to oncogenic mutation (Wan *et al.*, 2004). In contrast, the role of the amino-terminal B-Raf-specific region (called the BRS region hereafter) has not been elucidated.

We recently developed a probe, Prin-cRaf, based on the principle of fluorescent resonance energy transfer, to monitor the structural change of c-Raf. This probe has revealed that Ras binding to c-Raf induces open active conformation to facilitate binding to MEK (Terai and Matsuda, 2005). In the present study, we have developed a probe for B-Raf and found that B-Raf, but not c-Raf, homo-dimerizes upon Ras-dependent activation. The BRS region, which binds to the carboxy termini of B-Raf and c-Raf, is responsible for the dimerization. Furthermore, we found that the BRS region enhances MEK activation in a manner dependent on increases in the intracellular calcium concentration.

Results

Development of a probe to monitor the conformational change of B-Raf

To monitor the conformational change of B-Raf in living cells, we developed a probe named Prin-BRaf. Similar to Prin-cRaf (Terai and Matsuda, 2005), Prin-BRaf is comprised of yellow fluorescent protein (YFP), B-Raf, and cyan fluorescent protein (CFP) from the amino terminus (Figure 1A). We stimulated HeLa cells expressing Prin-BRaf and H-Ras with epidermal growth factor (EGF). In analogy to Prin-cRaf, we expected that upon Ras activation, the closed inactive form of Prin-BRaf would translocate to the plasma membrane and adopt an open active conformation, resulting in a decreased fluorescence resonance energy transfer (FRET) level. Upon EGF stimulation of HeLa cells, a major fraction of Prin-BRaf translocated to the plasma membrane, as expected. However, the FRET level increased rapidly, suggesting that the modes of action might differ significantly between c-Raf and B-Raf (Figure 1B and C, and Supplementary video 1). Similar experiments were performed with the Prin-BRafR188L

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mutant, in which Leu is substituted for Arg¹⁸⁸ within the Ras-binding domain. Prin-BRafR188L neither translocated to the plasma membrane nor exhibited change in the level of FRET, indicating that Ras binding was essential for the conformational change detectable with the Prin-BRaf probe (data not shown).

The most conspicuous structural hallmark of B-Raf is the B-Raf-specific amino-terminal extension of 116 amino acids, which is referred to as the BRS region. Taking this into account, the increase in the FRET level upon EGF stimulation could be attributable to any of three possibilities. First, the Ras-induced conformational change of B-Raf is entirely different from that of c-Raf, irrespective of the presence of the BRS region. Second, the BRS region renders the position of B-Raf's amino terminus remote to its carboxy terminus and brings the amino terminus close to the carboxy terminus in an EGF-dependent manner. The third possibility is that the BRS region induces head-to-tail dimerization of B-Raf in an EGF-dependent manner. We examined these possibilities by using three probes. One was Prin-BRaf Δ N, which lacked the BRS region, rendering the structure of this probe very similar to that of Prin-cRaf. The others were BRSR-Prin-BRaf Δ N and BRSR-Prin-cRaf, which contained the BRS region at the amino terminus of Prin-BRaf Δ N and Prin-cRaf, respectively (Figure 1A). Effect of EGF on FRET level was examined in cells expressing either of these probes (Figure 1D). When the BRS region was deleted (Prin-BRaf Δ N), the FRET efficiency was reduced to that of the cells expressing Prin-cRaf. This observation showed that B-Raf without the BRS region adopted an open active conformation in a Ras-dependent manner, as did the wild-type c-Raf. The addition of the BRS region to Prin-BRaf Δ N (BRSR-Prin-BRaf Δ N) restored the EGF-induced increase in the FRET level, strongly suggesting that the increase in FRET efficiency depended on the BRS region-induced dimerization. In contrast, the addition of the BRS region to Prin-cRaf (BRSR-Prin-cRaf) did not cause EGF-induced increase in FRET efficiency. This is probably because the affinity of the BRS region to c-Raf was lower than that to B-Raf. The decrease in FRET caused by the conformational change seemed to be larger than the increase in FRET caused by the head-to-tail dimerization mediated by the BRS region in the context of c-Raf.

To investigate the BRS region's role in B-Raf conformation more quantitatively, we utilized a FACS-based method as reported recently (Kawai *et al.*, 2004). In this method, probes were expressed with or without additional recombinant proteins in 293F suspension culture cells, in which the level of FRET was quantitated as described fully in Supplementary data. To rule out the possibility that the increase in FRET efficiency reflected concentration of probes, we analyzed cells expressing similar amount of probes (Supplementary Figure 2). In 293F cells expressing Prin-BRaf, the FRET level was increased only marginally by a constitutively active Ras mutant, RasV12, suggesting that the activation of Ras was not sufficient for the increase in FRET (Figure 1E). Ras affected the conformation of Raf proteins by either direct binding or an indirect mechanism following plasma membrane translocation (Terai and Matsuda, 2005). To distinguish between these two effects, we used the RasV12 Δ C mutant, which lacked the carboxy-terminal membrane-targeting signal and therefore was localized in the cytosol. This RasV12 Δ C mutant decreased the level of FRET, indicating that the full-length

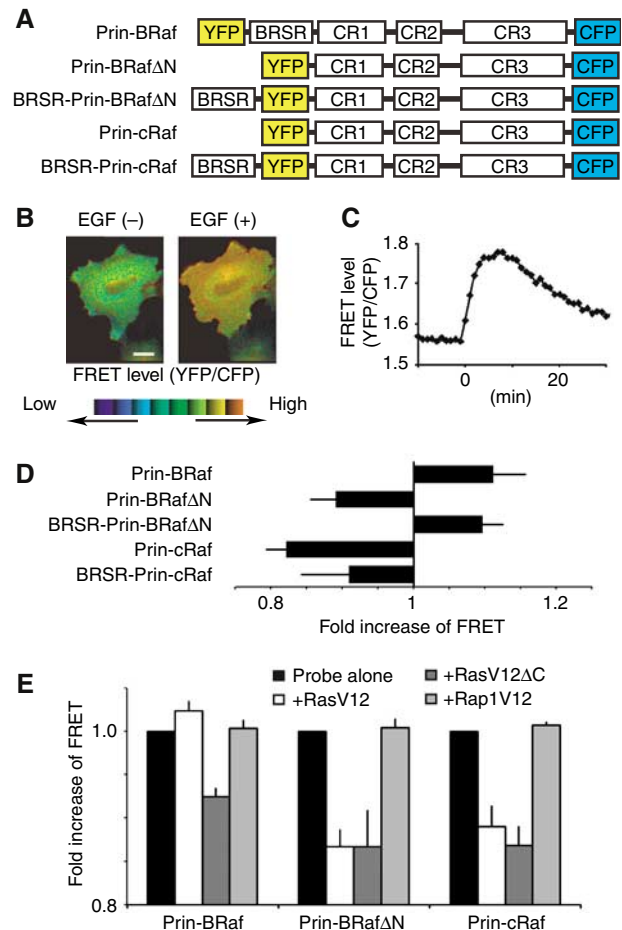


Figure 1 The BRS region increases the FRET level observed for the Prin-BRaf probe. (A) Schematic representation of FRET probes. BRSR: B-Raf-specific region; CR1: conserved region 1 including the Ras-binding domain and the cysteine-rich domain; CR2: conserved region 2; CR3: conserved region 3 including the catalytic domain. (B) HeLa cells coexpressing Prin-BRaf and H-Ras were stimulated with 25 ng/ml EGF and time-lapse imaged (Supplementary video 1). The FRET level (YFP/CFP) is shown in the intensity modulated display (IMD) mode. The scale bar shows 10 μ m. (C) Time courses of FRET level averaged for the cells overall are plotted against time. (D) HeLa cells expressing the FRET probes and H-Ras were stimulated with 25 ng/ml EGF. The fold increase in the FRET level (YFP/CFP) of the whole cell area 5 min after stimulation is shown. The bars and lines represent the average and the s.d., respectively ($N=5$). (E) 293F cells expressing the FRET probes with RasV12, RasV12 Δ C (a cytosolic mutant), or Rap1V12 were analyzed by FACS. The FRET level (YFP/CFP) relative to that of cells without any GTPases is shown. The bars and lines represent the average and the s.d., respectively ($N=5$).

B-Raf was induced to adopt an open active conformation by Ras binding. In this condition, Prin-BRaf Δ N behaved very similarly to Prin-cRaf: the FRET level was decreased by both RasV12 and RasV12 Δ C. We did not detect any effect of Rap1V12, an active mutant of Rap1, on the FRET level in cells expressing Prin-BRaf, Prin-BRaf Δ N, or Prin-cRaf. These observations indicated that B-Raf adopted open active conformations by binding to Ras, as did c-Raf. However, as shown in Figure 1D, when Prin-BRaf was translocated to the plasma membrane upon EGF stimulation, the FRET level was increased only when the BRS region was present. Thus, the level of FRET in cells expressing Prin-BRaf seemed to be

influenced by two independent mechanisms, one of which was common between B-Raf and c-Raf, the other mediated by the BRS region.

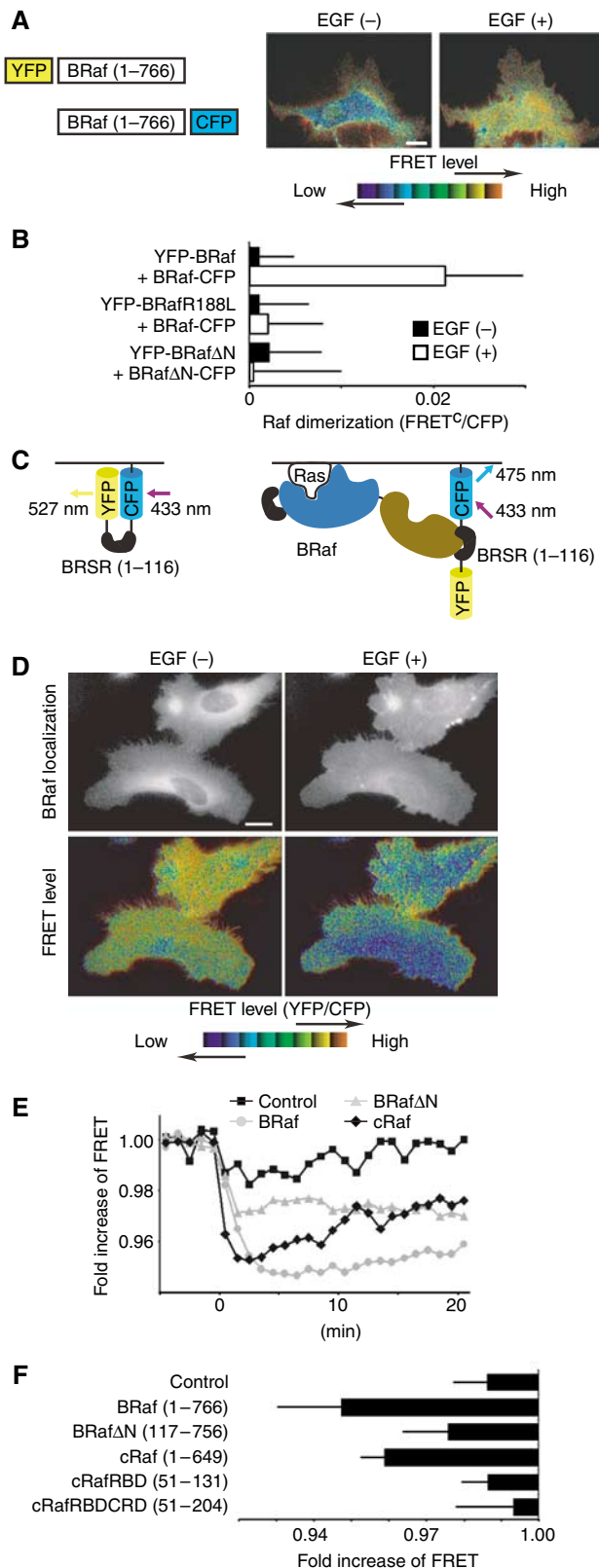
To examine whether or not the structural change of B-Raf without the BRS region was controlled in a manner similar to the structural change of c-Raf, we introduced a series of mutations into the Prin-BRaf Δ N background in the same way that we prepared Prin-cRaf previously (Terai and Matsuda, 2005), and examined the FRET level in the presence or absence of RasV12 Δ C. In brief, the results with the mutants indicated that the conformation of B-Raf without the BRS region seemed to be regulated very similarly to that of c-Raf (described in detail in Supplementary data and Supplementary Figure 1).

Dimerization of B-Raf and c-Raf mediated by the BRS region

Using a pair of YFP-BRaf and BRaf-CFP constructs, we next verified the idea that the increase in the FRET efficiency of Prin-BRaf is caused by head-to-tail dimerization (Figure 2A and B). EGF stimulation of HeLa cells expressing these two Raf probes induced remarkable increases in the FRET level, concomitant with translocation of the B-Raf proteins to the plasma membrane. Increase in the FRET level was not detected using the pair of YFP-BRafR188L/BRaf-CFP or YFP-BRaf Δ N/BRaf Δ N-CFP, indicating that Ras binding and the BRS regions are indispensable. When CFP and YFP were fused to the same side of B-Raf (YFP-BRaf and CFP-BRaf; BRaf-YFP and BRaf-CFP), the level of FRET did not change upon EGF stimulation (data not shown), indicating that the FRET increase was caused by the head-to-tail dimerization of B-Raf.

To examine the BRS region's role in B-Raf interaction more directly, we developed another probe, Prin-BRSR-pm. This probe is designed such that the level of FRET decreases when Prin-BRSR-pm binds to B-Raf, as is schematically shown in Figure 2C. To verify the probe's mode of action, we coexpressed Prin-BRSR-pm with RFP-BRaf and H-Ras in HeLa cells and stimulated them with EGF (Figure 2D and Supplementary video 2). Under these conditions, a major fraction of RFP-BRaf rapidly translocated to the plasma membrane, concomitant with a decrease in FRET efficiency, as expected. By using this probe, we determined the region required for

binding to the BRS region as carboxy-terminal of B-Raf (Figure 2E and F). An EGF-induced decrease in the FRET level was observed with B-Raf Δ N, supporting the idea of head-to-tail dimerization. Importantly, c-Raf was also found to decrease the FRET level, indicating that B-Raf could form



a hetero-dimer with c-Raf. These results suggested that the BRS region interacted with c-Raf and B-Raf at their catalytic domains.

B-Raf dimerization requires calcium signals

We further explored the mechanism of B-Raf dimerization and found that increases in intracellular calcium contributed to the B-Raf dimerization upon EGF stimulation. Using the calcium sensor, Fura-2/AM, we confirmed that EGF-dependent increases in calcium were mediated primarily by PLC- γ , because a dominant-negative mutant of PLC- γ , PLC-223, completely suppressed EGF-induced calcium increases (Figure 3A). In HeLa cells expressing Prin-BRaf and PLC-223, the EGF-induced increase in the level of FRET was abrogated, although Prin-BRaf translocated to the plasma membrane in PLC-223-expressing cells, as in the control cells (Figure 3B–D and Supplementary video 3). In fact, in HeLa cells expressing PLC-223, the FRET level decreased upon EGF stimulation. To further explore the role of calcium, we used the intracellular Ca^{2+} chelator BAPTA/AM and the selective PKC inhibitor Go 6850. In HeLa cells, the elevation of the FRET efficiency of Prin-BRaf was abrogated by BAPTA/AM but not by Go 6850. Notably, neither PLC-223 nor BAPTA/AM affected Ras activation, as demonstrated by the clear observation of translocation of Prin-BRaf to the plasma membrane. These results indicated that B-Raf dimerization at the plasma membrane required increases in intracellular calcium. Finally, we tested whether or not an influx of calcium would induce B-Raf dimerization in the presence of active Ras (Figure 3E). Ionomycin increased the FRET of cells expressing Prin-BRaf and RasV12 but not in cells expressing Prin-BRaf and RasV12 Δ C. This observation strongly supported our view that the increases in intracellular calcium trigger the formation of B-Raf dimers at the plasma membrane.

Modulation of calcium-stimulated MEK phosphorylation by the BRS region

To investigate the role of the BRS region in a physiologic milieu, we generated stable HeLa cell lines expressing either B-Raf, B-Raf Δ N, or c-Raf at a level comparable to the endogenous Raf proteins. The concentrations of endogenous and exogenous Raf proteins were determined by quantitative immunoblotting. In the parent HeLa cells, the concentrations of endogenous B-Raf and c-Raf were 0.0035 and 0.013 μM , respectively (Fujioka *et al.*, 2006) (Supplementary Figure 4). The concentration of both exogenous B-Raf and B-Raf Δ N was 0.018 μM . Thus, the concentration of exogenous B-Raf was comparable to that of the endogenous c-Raf in the HeLa cell lines used in the following experiments. These HeLa cell lines were stimulated with various reagents and examined for the activation levels of endogenous MEK and ERK (Figure 4A and B). We found that ionomycin-induced activation of MEK and ERK was remarkably enhanced by the expression of B-Raf but not by that of B-Raf Δ N or c-Raf. Recently, it has been shown that B-Raf activates ERK via c-Raf and that the catalytic activity of B-Raf is dispensable for this pathway (Garnett *et al.*, 2005; Rushworth *et al.*, 2006). In agreement with this observation, we found that expression of a kinase-negative mutant of B-Raf also enhanced ionomycin-induced ERK phosphorylation (data not shown). B-Raf-Arg¹⁸⁸Leu did not remarkably enhance ionomycin-induced MEK phos-

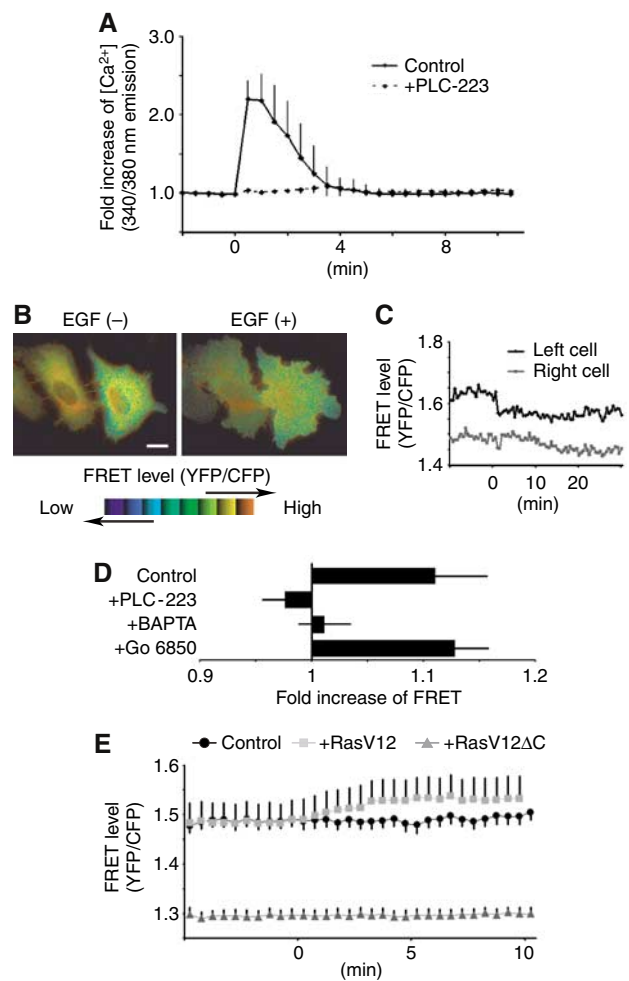
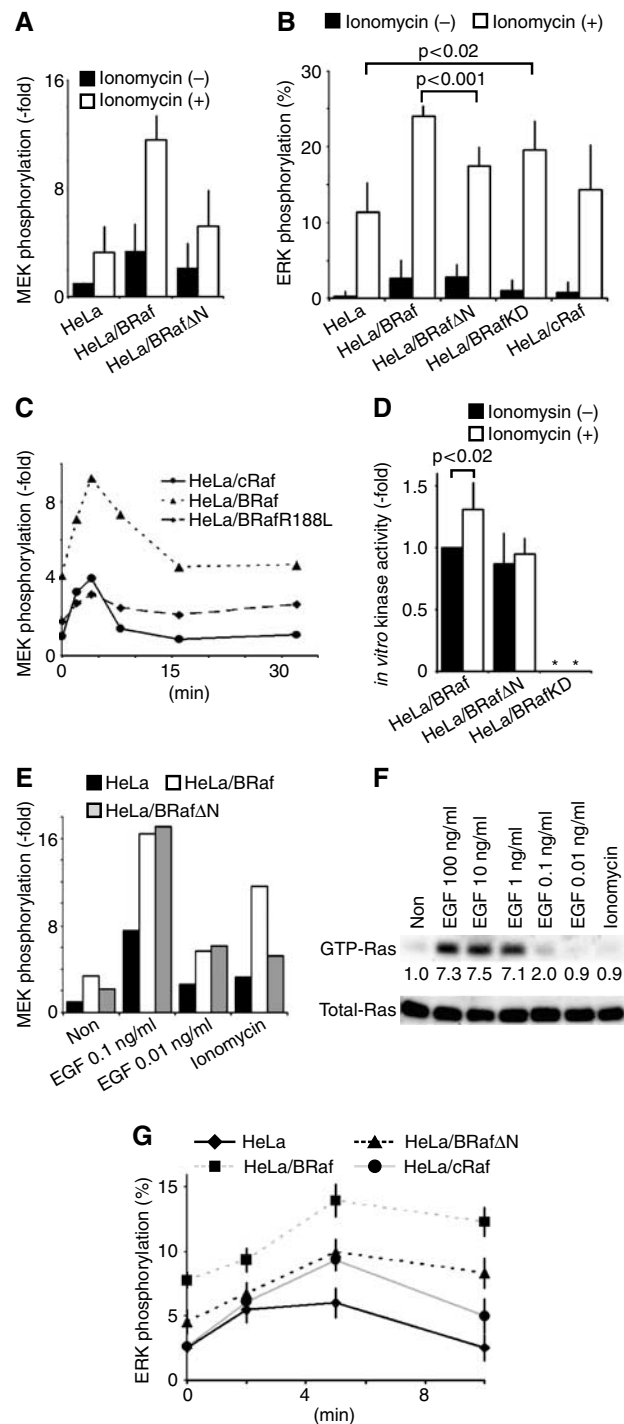


Figure 3 B-Raf dimerization requires increases in calcium and plasma membrane translocation. (A) HeLa cells loaded with a calcium indicator Fura-2 were stimulated with 25 ng/ml EGF in the presence or absence of a dominant-negative mutant of PLC- γ , PLC-223. Dual-excitation (340 nm/380 nm) ratio imaging was performed to monitor the level of intracellular free-calcium concentration. Time courses of the fold increases of emission ratio (340 nm/380 nm) averaged over the cells are plotted against time. The bars represent the s.d. ($N=5$). (B) HeLa cells coexpressing Prin-BRaf, H-Ras, and PLC-223 were stimulated with 25 ng/ml EGF and time-lapse imaged (Supplementary video 3). Representative FRET images are shown in the IMD mode. (C) Time courses of the FRET level (YFP/CFP) averaged for the cells overall are plotted against time. (D) HeLa cells expressing Prin-BRaf and H-Ras were stimulated with 25 ng/ml EGF in the presence or absence of a calcium chelator BAPTA/AM (30 μM) or a PKC inhibitor Go 6850 (100 nM). The fold increase in the FRET level (YFP/CFP) of the whole cell area at 5 min after stimulation is shown. (E) HeLa cells expressing Prin-BRaf alone or with H-RasV12 or H-RasV12 Δ C were stimulated with 1 μM ionomycin. Time courses of FRET level (YFP/CFP) averaged for the cells are plotted against time. The bars represent the s.e.m. ($N=5$).

phorylation (Figure 4C), indicating that the calcium-induced B-Raf activation was Ras-dependent. The calcium-dependent B-Raf activation was also examined *in vitro* (Figure 4D). Ionomycin activated wild-type B-Raf but not B-Raf Δ N, confirming that the BRS region is required for the calcium-dependent B-Raf activation. It is worth noting that no such BRS region-dependent enhancement of MEK activation was observed in cells stimulated with decreasing concentrations of EGF (Figure 4E). Notably, calcium stimulation did not increase the level of GTP-Ras in HeLa cells to a detectable

level (Figure 4F). These results suggested that the BRS region mediates the calcium-dependent B-Raf activation even in conditions where GTP-Ras level is not increased. Next, we tested whether the BRS region contributes to muscarinic receptor-dependent ERK stimulation because a previous report shows that heterotrimeric G-protein-coupled receptors induce Raf activation through a calcium-dependent pathway (Della Rocca *et al.*, 1997). HeLa cell lines stably expressing B-Raf, B-Raf Δ N, or c-Raf were stimulated with carbachol and examined for the phosphorylation of ERK (Figure 4G). Carbachol-dependent activation of ERK was more potently enhanced by wild-type B-Raf than by B-Raf Δ N or c-Raf.



These results supported our model that the BRS region contributes to the activation of the MEK-ERK pathway in a calcium-dependent manner.

B-Raf dimerization induces phosphorylation of Thr¹¹⁸

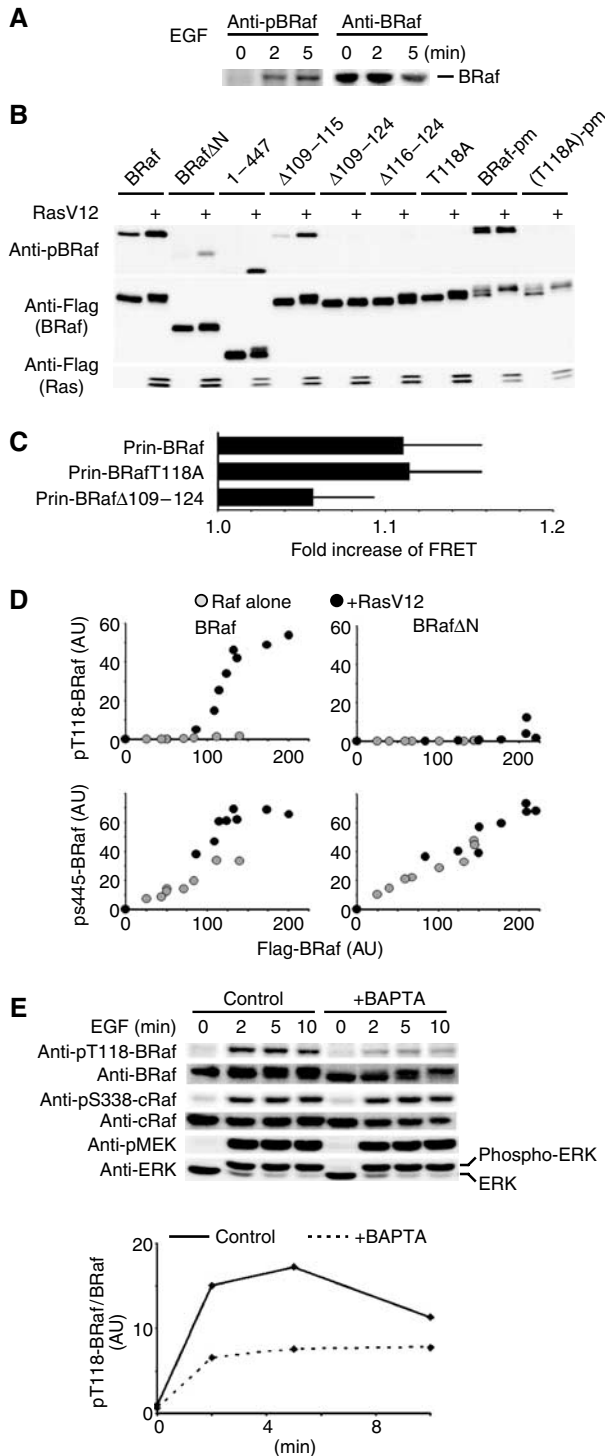
Phosphorylation plays a pivotal role in the regulation of the catalytic activities of many serine threonine kinases. In particular, the phosphorylation of amino acids within the catalytic loop has been shown to play the essential role of the kinase activation (Huse and Kuriyan, 2002). To investigate the role of phosphorylation in dimerization, we immunized four rabbits with the phosphorylated peptides within the B-Raf catalytic loop [NH₂-CGLApT⁵⁹⁸TVKpS⁶⁰¹RWSG-COOH] and obtained rabbit sera that specifically detected endogenous B-Raf in Cos7 cells stimulated with EGF (Figure 5A). Unexpectedly however, the rabbit sera were found to detect T598A/S601A and T598E/601D mutants as efficiently as wild type in 293F cells expressing RasV12 (Supplementary Figure 3A). Further mutagenic analyses revealed that the anti-phospho-B-Raf sera primarily recognized phosphorylated Thr¹¹⁸ within the amino-terminal serine-rich region (Figure 5B and Supplementary Figure 3B). In addition, it was also observed that membrane targeting of B-Raf was sufficient for the phosphorylation of this residue (Figure 5B and Supplementary Figure 3B). Although it was unknown why the rabbit sera raised against the catalytic loop recognized the peptide sequence in the amino-terminal regulatory region, excellent correlation of the appearance of this epitope with the activation of B-Raf urged us to further characterize this serum.

A B-Raf Δ N construct, which was unable to dimerize, yet retained Thr¹¹⁸, was phosphorylated significantly less than wild-type B-Raf; hence, we considered the following two possibilities: phosphorylation of Thr¹¹⁸ is required for dimerization, or B-Raf dimerization is required for phosphorylation of Thr¹¹⁸. The first possibility was negated because the EGF-induced increase in the FRET level was observed in HeLa cells expressing Prin-BRafT118A or Prin-BRaf Δ 109-124 (Figure 5C). To test the latter possibility, we examined the correlation of B-Raf concentration versus phosphorylation

Figure 4 The BRS region enhances calcium-induced MEK phosphorylation. (A, B) HeLa-derived cell lines expressing Raf proteins as indicated were stimulated with 1 μ M ionomycin and lysed at 5 min. BRaf Δ N, a B-Raf mutant without the BRS region; BRafKD, a catalytically inactive mutant. Cell lysates were analyzed by immunoblotting with anti-phospho-MEK antibody (A) or anti-ERK antibody (B). The intensity ratio of the slow-migratory form and the rapid-migratory form of endogenous ERK2 on anti-ERK blot was used to calculate the molar ratio of phosphorylated versus non-phosphorylated ERK2. The bars and lines represent the average and the s.d., respectively ($N \geq 3$). Similar results were obtained using three independent cell lines that express comparable levels of B-Raf. (C) Cells were stimulated with 1 μ M ionomycin and analyzed at the indicated time points for MEK phosphorylation. (D) Cells were stimulated with 1 μ M ionomycin and lysed at 5 min. Flag-BRaf, Flag-BRaf Δ N, and Flag-BRafKD were immunoprecipitated from cell lysates and analyzed *in vitro* for the MEK kinase activity ($N \geq 3$). (E) Cells were stimulated with decreasing amounts of EGF or 1 μ M ionomycin for 5 min and analyzed for the level of MEK phosphorylation. (F) HeLa cells were stimulated with decreasing amounts of EGF or 1 μ M ionomycin for 5 min and analyzed for GTP-Ras level by Bos' pull-down method. (G) Cells were stimulated with carbachol (100 μ M) and analyzed for the level of ERK phosphorylation. The bars represent the s.e.m. ($N = 3$).

level in cells expressing a saturating amount of RasV12. We assumed that, if the Thr¹¹⁸ phosphorylation depended solely on the RasV12-dependent membrane translocation, the concentrations of B-Raf would correlate linearly with the level of Thr¹¹⁸ phosphorylation. Meanwhile, if it depended on the dimer formation, high concentration would accelerate the phosphorylation of Thr¹¹⁸. As shown in Figure 5D, the level of Thr¹¹⁸ phosphorylation showed a sigmoidal increase with the increase in the concentration of B-Raf, supporting the latter possibility. The linearity of the detection with anti-

pThr¹¹⁸ B-Raf antibody was verified by showing that serially diluted samples from the same cell lysates demonstrated a linear decrease in the intensity (Supplementary Figure 3C). Furthermore, in a clear contrast to Thr¹¹⁸ phosphorylation, the level of Ser⁴⁴⁵ phosphorylation correlated linearly with the B-Raf concentration, indicating that phosphorylation of this residue was independent of B-Raf dimerization. As expected, because of the dependence of B-Raf dimer formation on calcium, we found that the Thr¹¹⁸ phosphorylation was significantly impaired by the intracellular Ca²⁺ chelator BAPTA/AM (Figure 5E). In the same condition, the phosphorylation or activation of c-Raf, MEK, and ERK was not affected. These data strongly supported our proposal that B-Raf dimerization induces the phosphorylation of Thr¹¹⁸.



Increase in the intracellular calcium concentration induces B-Raf translocation to the plasma membrane

Finally, we examined whether or not increases in the level of intracellular calcium concentration were sufficient to induce B-Raf translocation to the plasma membrane. For this purpose, we established a semiquantitative assay for the plasma membrane translocation. Owing to the bell-shaped morphology of the adherent cells, plasma membrane translocation of cytosolic fluorescent proteins is manifested as an increase in the fluorescence at the peripheral region of the cells and a decrease in the perinuclear region. By using a red fluorescent protein fused to the nuclear export signal as an internal reference, translocation of GFP-tagged proteins was quantified to obtain a translocation index. With this method, EGF-dependent c-Raf translocation to the plasma membrane could be quantitatively followed (Supplementary Figure 5 and Supplementary video 4). Similarly, but to a lesser extent, ionomycin induced plasma membrane translocation of B-Raf (Figure 6 and Supplementary video 5). B-Raf without Ras-binding domain and c-Raf did not respond to ionomycin. Removal of the BRS region significantly attenuated the ionomycin-induced plasma membrane translocation. On the other hand, amino-acid substitution of Thr¹¹⁸ did not affect the ionomycin-dependent plasma membrane translocation. Thus, the BRS region and Ras-binding region are indispensable, but the phosphorylation of Thr¹¹⁸ in the BRS

Figure 5 Phosphorylation of Thr¹¹⁸ of B-Raf requires B-Raf-specific region and calcium signal. (A) Cos7 cells before and after EGF stimulation were lysed and analyzed by immunoblotting with anti-phospho-B-Raf (anti-pBRaf) or anti-B-Raf antibody (anti-BRaf). (B) 293F cells expressing B-Raf with or without H-RasV12 were analyzed by immunoblotting with anti-phospho-B-Raf or anti-Flag antibody. (C) HeLa cells expressing the FRET probes indicated on the left and H-Ras were stimulated with 25 ng/ml EGF. The fold increase in the FRET level (YFP/CFP) of the whole cell area at 5 min after stimulation ($N=5$) is shown. (D) 293F cells (5×10^5) transfected with increasing amounts of B-Raf or B-RafΔN expression plasmids (from 0.005 to 0.2 μg) in the presence or absence of 0.2 μg H-RasV12 expression plasmid were analyzed by SDS-PAGE and immunoblotting with anti-pBRaf antibody, anti-pSer³³⁸ c-Raf, or anti-Flag antibody. Note that anti-pSer³³⁸ c-Raf antibody cross-reacts with pSer⁴⁴⁵ B-Raf. (E) Cos7 cells were stimulated with 25 ng/ml EGF for the indicated periods of time in the presence or absence of BAPTA/AM (30 μM). Cell lysates were analyzed by immunoblotting with anti-phospho-B-Raf (pT118), anti-B-Raf, anti-pSer³³⁸ c-Raf (pS338), anti-c-Raf, anti-phospho-MEK (pMEK), or anti-ERK antibody. The ratio of phospho-B-Raf to B-Raf is plotted against time.

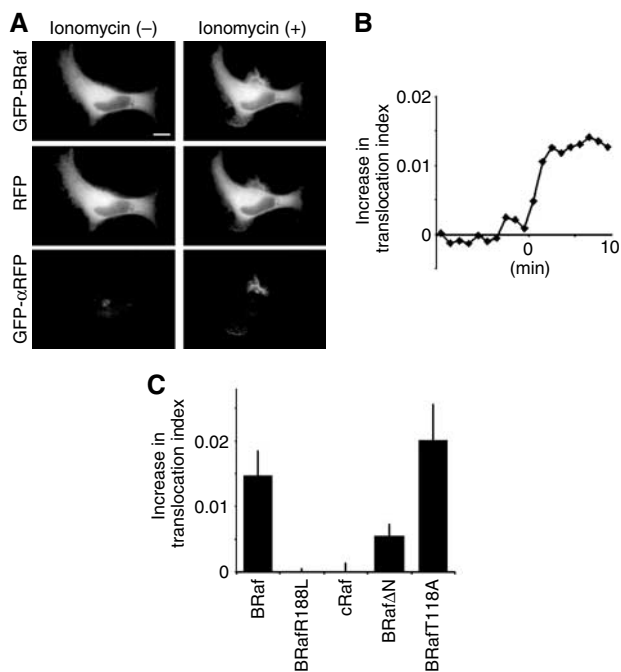


Figure 6 Plasma membrane translocation of B-Raf by calcium agonist. **(A, B)** HeLa cells expressing GFP-BRaf, H-Ras, and RFP-NES were stimulated with 1 μ M ionomycin and time-lapse imaged (A). The difference in the distribution of B-Raf and RFP-NES, the latter of which is used as a reference of cytosolic protein, was imaged as GFP- α RFP and quantified to obtain 'translocation index' (described in Supplementary data). Time courses of the translocation index are plotted against time (B). **(C)** HeLa cells expressing H-Ras with GFP-BRaf, GFP-BRafR188L, GFP-cRaf, GFP-BRaf Δ N, or GFP-BRafT118A were stimulated with 1 μ M ionomycin. The fold increase in the translocation index 5 min after stimulation is shown. The bars and lines represent the average and the s.e.m., respectively ($N \geq 5$).

region is dispensable for the calcium-dependent membrane translocation of B-Raf.

Discussion

This study has shown that the BRS region mediates calcium-dependent homo-dimerization of B-Raf and hetero-dimerization of B-Raf and c-Raf and the following Thr¹¹⁸ phosphorylation. The hetero-dimerization of B-Raf and c-Raf was already described by Weber *et al* (2001) and has been suggested to be critical in oncogenesis mediated by mutated B-Raf proteins in human melanoma (Wan *et al*, 2004). Furthermore, it has been shown that the catalytic activity of B-Raf is dispensable for the ERK activation (Garnett *et al*, 2005; Rushworth *et al*, 2006). In agreement with these reports, we found that a kinase-negative B-Raf also enhanced calcium-dependent ERK activation (Figure 4B).

It is still controversial whether Ras is required for the dimerization of B-Raf and c-Raf. Weber *et al* (2001) have shown that Ras induces hetero-dimerization of B-Raf and c-Raf. However, Garnett *et al* (2005) have shown that the hetero-dimerization of B-Raf and c-Raf could be formed in the cytosol in a Ras-independent manner. Our results obtained with FRET probes are consistent with the report by Weber *et al* (2001) in that Ras is required for dimerization and that the carboxy half of c-Raf binds to B-Raf. Furthermore, by the

use of a FRET probe consisting solely of the BRS region, we have shown that the dimer is formed in a head-to-tail manner (Figure 2). Based on our previous and present data, we propose that only Raf proteins in an open active conformation are able to dimerize. This model may explain the requirement of Ras binding for the dimerization of Raf proteins as has been reported previously (Weber *et al*, 2001) and in this study. Notably, by the co-immunoprecipitation method, we could not observe any dependency of the dimerization of B-Raf and c-Raf on either Ras or the BRS region (Supplementary Figure 6). This observation agrees with the report by Garnett *et al* (2005) and highlights the difference in the methods to detect dimerization. Whereas the co-immunoprecipitation method detects the amount of dimerization, the FRET-based method detects the ratio of dimerized versus monomeric Raf proteins. Therefore, the sensitivity of the FRET-based method is significantly lower than that of the co-immunoprecipitation method, when the proportion of the dimerized form is markedly lower than that of the monomeric form. It is likely that the regulatory regions of Raf proteins, which bind to phospholipids (Mott *et al*, 1996), may also contribute to the stability of the B-Raf dimer in living cells. If so, the membrane-bound RasV12 may induce dimerization more effectively at the plasma membrane than does the cytosolic RasV12 mutant.

The dimerization of Raf proteins has been suggested to elevate their kinase activity. For example, induced dimerization of c-Raf has been shown to elevate its kinase activity (Farrar *et al*, 1996; Luo *et al*, 1996), although homo-dimerization of c-Raf has not been demonstrated in any physiological content. Additionally, B-Raf-mediated c-Raf activation has been demonstrated both *in vitro* (Mizutani *et al*, 2001) and *in vivo* (Wan *et al*, 2004). These observations support our proposal that the BRS region plays a critical role in the activation of Raf proteins by means of inducing B-Raf dimerization.

The mechanism underlying calcium-induced B-Raf dimerization and the resulting Thr¹¹⁸ phosphorylation is currently unknown. EGF-induced B-Raf dimerization continues for some 30 min in HeLa cells, whereas in the same condition the calcium spike lasts at most 4 min (Figure 4). Thus, it is likely that B-Raf dimerization and Thr¹¹⁸ phosphorylation are modulated by calcium-activated signaling molecules such as Ca²⁺/calmodulin-dependent protein kinase II (CaMKII). In support of this idea, the requirement of CaMKII for Raf activation has been demonstrated in nerve growth factor-stimulated PC12 neuronal cells (Egea *et al*, 2000).

The level of phosphorylated Thr¹¹⁸ was correlated positively with B-Raf activation by EGF stimulation, RasV12 expression, and plasma membrane anchoring. However, as far as we have examined, single amino-acid substitution of Thr¹¹⁸ does not affect any function of B-Raf. Because there are nine Ser/Thr residues between Ser¹⁰⁹ and Ser¹²⁴, we speculate that in the Thr¹¹⁸Ala mutant, phosphorylation of other Ser/Thr residue may occur to compensate for loss of phosphorylated Thr¹¹⁸. The observation that Ras-induced increase in FRET is attenuated in Prin-BRaf Δ 109–124 (Figure 5C) suggests that phosphorylation of this region promotes the dimerization of B-Raf.

Various ligands activate the ERK MAP kinase in a calcium-dependent manner (Chao *et al*, 1992): for example, ERK activation by Gq-dependent pathway is mostly dependent

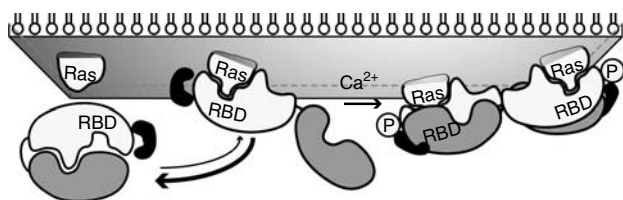


Figure 7 A novel model of calcium-induced B-Raf activation. In resting cells, B-Raf is in equilibrium between a small fraction of Ras-bound open monomeric state and a large fraction of cytosolic monomeric state. Increase in the cytoplasmic calcium concentration stabilize the B-Raf-Ras complex by induction of dimerization. The increase in the B-Raf-Ras complex at the plasma membrane enhances MEK phosphorylation, thereby activating ERK MAP kinase.

on calcium influx (Eguchi *et al*, 1996; Della Rocca *et al*, 1997; Strakova *et al*, 1998; Suarez *et al*, 2003; Chan *et al*, 2005). However, the mode of action of calcium may vary significantly depending on cell type and the particular stimulation. In PC12 cells, activation of Gq-coupled muscarinic receptor triggers calcium influx and thereby activates CalDAG-GEF1/RasGRP2, a guanine nucleotide factor for Rap1, followed by B-Raf activation by Rap1 (Guo *et al*, 2001). In the pancreatic cell line MIN6, it is proposed that calcium influx induces ERK activation independent of the activation of Raf and Ras (Gomez *et al*, 2002). In HeLa and Cos7 cells used in the present study, treatment with a calcium ionophore did not stimulate either Ras (Figure 4D) or Rap1 (data not shown) to a detectable level. Notably, we found that expression of B-Raf, but not c-Raf or B-Raf without the BRS region, enhanced MEK activation in cells stimulated with calcium ionomycin or carbachol (Figure 4). Based on these observations, we propose that the calcium-dependent signaling pathway in Cos7 cells is merged primarily at the level of B-Raf, probably by means of dimerization and the following Thr¹¹⁸ phosphorylation.

To consider the functional role of B-Raf dimerization and the resulting Thr¹¹⁸ phosphorylation, it is extremely important to quantify the intracellular concentrations of Ras and B-Raf. The concentration of Ras (H-, N-, and K-) is 0.40 μM in HeLa cells and 0.53 μM in Cos7 cells; that of c-Raf is 0.013 μM in HeLa cells and 0.005 μM in Cos7 cells; that of B-Raf is 0.0035 μM in HeLa cells and 0.006 μM in Cos7 cells (Fujioka *et al*, 2006) (Supplementary Figure 4). Because the GTP/GDP

level of Ras in the quiescent state is between 1 and 20% in many cell types (Sato *et al*, 1991; Carter *et al*, 1995; Zheng *et al*, 1997; de Rooij and Bos, 1997; Ohba *et al*, 2003), the net amount of GTP-Ras should be sufficient to recruit B-Raf proteins to the plasma membrane, only if the affinity of B-Raf to Ras is stronger than that of the other Ras effector molecules. Thus, even though we could not detect Ras activation in calcium agonist-stimulated cells either with FRET probes or in the pull-down assay (Figure 4F), B-Raf can be recruited to Ras if the binding between Ras and B-Raf is strengthened. The requirement of the Ras-binding domain of B-Raf for the calcium-induced activation of ERK is fully consistent with this model (Figure 4C). We speculate that B-Raf dimerization and the following Thr¹¹⁸ phosphorylation may increase the affinity of B-Raf to GTP-Ras, thereby transmitting the signals to a level above that generated by basal GTP-Ras in the absence of calcium signals.

In conclusion, we propose a novel signaling mechanism wherein B-Raf could be activated without an increase in Ras-GTP. Upon an increase in the intracellular calcium concentration, the BRS region of B-Raf induces B-Raf dimerization and the following phosphorylation of the BRS region, which probably stabilizes B-Raf binding to Ras (Figure 7). Considering the abundance of B-Raf in neuronal cells, this calcium-dependent regulatory mechanism suggests that the MEK-ERK cascade may be finely controlled by neuronal activity via this calcium-B-Raf pathway.

Materials and methods

See Supplementary data for details.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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