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A Bimolecular Fluorescent Complementation Screen Reveals Complex Roles of Endosomes in Ras-Mediated Signaling

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Abstract

While Ras GTPases are best known for mediating growth factor signaling on the plasma membrane, these proteins also have surprisingly complex activities in the endosome. Assisted by a method called bimolecular fluorescent complementation (BiFC), which can detect weak and transient protein–protein interactions and reveal where the binding takes place in live cells, we have identified three effectors, Cdc42, CHMP6, and VPS4A that interact with Ras proteins in endosomes. These effectors are all necessary for Ras-induced transformation, suggesting that for Ras proteins to efficiently induce tumor formation, they must also activate effectors in cytoplasm, such as those in endosomes. Here, we describe how BiFC can be used to detect and screen for Ras effectors and for readily revealing where in the cell the binding occurs.

1. INTRODUCTION

1.1. Ras protein and growth factor signaling

Ras proteins cycle between the GDP- and GTP-bound states to function as binary switches in order to mediate a wide range of signaling events. The best-known activity of the Ras proteins is mediating extracellular proliferative signals controlled by receptor tyrosine kinases (RTKs) in response to growth factors, such as EGF (epidermal growth factor). Deregulations in these signaling pathways have been linked to the development of many human cancers (Pylayeva-Gupta, Grabocka, & Bar-Sagi, 2011). Evidently, the growth factor signaling initiates at the plasma membrane to activate Ras proteins; however, what happens next to these Ras proteins on the plasma membrane is poorly understood.

1.2. RTK and Ras internalization

Internalizing receptors with seven transmembrane helices via endocytosis is a well-established mechanism for attenuating signals transduced by trimeric G proteins. As it turns out, receptor internalization appears to be a very common mechanism in reducing signaling outputs at the plasma membrane, as many RTKs, such as EGFRs (EGF receptors), are also internalized (Sigismund et al., 2012).

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While Ras proteins are well known for lipidations (farnesylation and palmitoylation) at the C-terminus to influence how they associate with various membrane compartments, in a surprising study by Jura, Scotto-Lavino, Sobczyk, and Bar-Sagi (2006), H- and N-Ras proteins have been found to be also ubiquitinated via a K-63 linkage. This mode of ubiquitination is often required for endocytosis and endosomal sorting, and indeed, we and others have found that these two Ras proteins frequently localize to the endosomes (Cheng & Chang, 2011; Cheng et al., 2011; Fehrenbacher, Bar-Sagi, & Philips, 2009; Zheng, Cheng, et al., 2012; Zheng, Xu, Bar-Sagi, & Chang, 2012).

While many Ras proteins enter the endosomes, the complex fates of these internalized Ras proteins are just beginning to resolve. When the lysine residues in the Ras proteins are mutated (Jura et al., 2006; Zheng, Cheng, et al., 2012) or when an ubiquitin ligase responsible for Ras ubiquitination (Rabex-5) is repressed (Xu, Lubkov, Taylor, & Bar-Sagi, 2010; Yan, Jahanshahi, Horvath, Liu, & Pfleger, 2010), Ras signaling outputs are apparently enhanced. Conversely, when Rabex-5 is overexpressed, the Ras signaling outputs are inhibited. These results suggest that Ras internalization can lead to attenuation of Ras-mediated signaling at the plasma membrane. Furthermore, one of the Ras effectors is RIN1 (Han et al., 1997), a guanine nucleotide exchange factor for Rab5, which controls endocytosis (Tall, Barbieri, Stahl, & Horazdovsky, 2001). Thus, it seems that Ras proteins can promote their own internalization by activating RIN1 in an apparent negative feedback loop. While this model explains why some of the Ras proteins are internalized, it does not appear to explain observations made by us (see in the succeeding text) and by others that endosomal Ras proteins are in fact functional and stimulate a specific set of effectors to induce transformation.

1.3. Ras interaction with Cdc42 in the endosome is required for efficient transformation

We and others have initially used the fission yeast *Schizosaccharomyces pombe* as a model system to study Ras functions (Chang et al., 1994; Chang & Philips, 2006; Fukui & Yamamoto, 1988; Onken, Wiener, Philips, & Chang, 2006). *S. pombe* has a single Ras protein, Ras1, that controls two spatially segregated pathways. Ras1 controls a protein kinase called Byr2, which activates a MAP kinase module at the plasma membrane to mediate signaling carried by the mating pheromone. Furthermore, Ras1 activates Cdc42 in the endomembrane via its guanine nucleotide exchange factor Scd1/Ral1 to mediate cell polarity to maintain an elongated cell morphology.

In a more recent study, we investigated whether the Ras–GEF–Cdc42 pathway has been conserved evolutionarily in mammalian cells (Cheng & Chang, 2011; Cheng et al., 2011). Indeed, we demonstrated that H-Ras, in a GTP-dependent manner, binds Cdc42 in the endosome, and Cdc42 is required for cellular transformation induced by oncogenic H-Ras. Activation of Ras in turn induces activation of Cdc42 apparently by recruiting and/or activating several guanine nucleotide exchange factors for Cdc42.

1.4. Using BiFC to seek and study new Ras effectors

To our knowledge, we were the first to demonstrate that Ras and Cdc42 can form a complex. We believe that many, including us, have tried using more conventional methods, such as

immunoprecipitation, to measure the binding and found that it was not easy to do so because the binding is weak and transient. We overcame this difficulty by employing a technology called bimolecular fluorescent complementation (BiFC) (Hu, Chinenov, & Kerppola, 2002).

In BiFC (Hu et al., 2002), one protein is fused to an N-terminal fragment of YFP (Yn, Fig. 2.1A), while its binding partner is fused to an C-terminal fragment of YFP (Yc, Fig. 2.1A). When the proteins of interest form a complex, the fragments of the fluorescent protein also refold to restore fluorescence, which can be readily detected by both FACS (Fig. 2.1B) and fluorescence microscopy (Fig. 2.1C). The BiFC method can detect weak and transient binding because it can “capture” a steady pool of proteins that bind one another, even though dynamic and/or transient protein binding often has a high off-rate at steady state. Moreover, as the two fragments of a fluorescent protein refold, extensive H-bonding between them further stabilizes the protein complex. In addition to being very sensitive, the BiFC system can readily reveal where the binding occurs in live cells. This latter property is particularly critical for the study of compartmentalized Ras signaling.

1.5. Promoting Ras recycling and transformation by components of the ESCRT-III complex

Since there is clear evidence that Ras proteins can interact with Cdc42 in a cell compartment-specific manner, we argue that it is important to understand this process thoroughly by systematically determining where in the cell a particular Ras–effector pair interacts. We are particularly interested in knowing how often a given Ras protein interacts with effectors in the cytoplasm, a cell compartment that is the least understood in the study of Ras signaling. In a recent proof-of-principle study, we screened human cDNA libraries using BiFC to identify Ras effectors that interact with a Ras protein selectively in the cytoplasm, but not on the plasma membrane (Zheng, Cheng, et al., 2012). Our screen uncovered a known Ras effector, A-Raf, plus over 20 new effectors with an intriguing enrichment in proteins known to mediate intracellular trafficking. Two of them, in particular, CHMP6/VPS20 and VPS4A, are well known to interact with the ESCRT-III (Endosomal Sorting Complex Required for Transport-III) complex (Raiborg & Stenmark, 2009). These two proteins were chosen for detail analysis.

A key role of the ESCRT-III complex is to promote protein sorting by controlling scission of the ubiquitylated cargo-containing buds in the endosomes to form intraluminal vesicles, which is a critical step of multivesicular body biogenesis (Hurley & Hanson, 2010). In our study, CHMP6 and VPS4A act like typical Ras effectors, in that they bind H-Ras directly in a GTP-dependent manner. More importantly, when their levels are reduced, Ras-induced cell transformation is attenuated. This phenomenon correlates with the loss of EGFR recycling, suggesting that Ras can act through these two effectors to promote RTK recycling back to the plasma membrane. The binding to CHMP6 and VPS4A also appears to require Ras ubiquitylation, suggesting that Ras proteins themselves act as cargoes when passing through the endosomes. To investigate this possibility further, we examined biochemically the portion of Ras in the cytoplasm versus that on the plasma membrane and found that the latter was reduced when the expression of either CHMP6 or VPS4A was repressed. We then carried out photobleaching experiment and the data show that reducing either CHMP6 or

VPS4A decreases the movement of Ras from the cytoplasm to the plasma membrane. These data collectively support the model that CHMP6 and VPS4A, if not the whole ESCRT-III complex, are responsible for recycling components of the Ras pathway (e.g., Ras proteins and RTKs) back to the plasma membrane to continue supporting the signal for growth. Thus, while internalized Ras proteins can remain dormant, some of them can also activate a recycling mechanism, presumably during persistent stimulation by the growth factors, in an apparent positive feedback loop for sustained and prolonged growth factor signaling (Zheng, Cheng, et al., 2012; Zheng, Xu, et al., 2012). In the succeeding text, we will describe in detail the methodology of using BiFC to study Ras–effector interactions.

2. DETECT RAS–EFFECTOR INTERACTIONS BY BiFC

2.1. General considerations for using BiFC to study Ras

While a large and growing list of fluorescent proteins have been reported to work efficiently in the BiFC system (Kodama & Hu, 2012), YFP and its derivatives remain popular choices because they have relative high quantum yields and complementation efficiencies among the GFP variants. In addition, several mutations have been introduced into the original YFP to accelerate maturation, which leads to the production of brighter YFP proteins (Kodama & Hu, 2012). We have chosen the “Venus” version of the YFP (Nagai et al., 2002), which contains a F46L mutation in the backbone of the “super”-EYFP (SEYFP) (Sawano & Miyawaki, 2000). The Venus is not only brighter than SEYFP but also more resistant to acid and Cl[−], which are common in many cellular compartments. In our study (Chen, Liu, & Songyang, 2007), the Yn is an N-terminal fragment of Venus, truncated at residue 155, a common split site for BiFC (Kodama & Hu, 2012). Since the majority of the “enhancement” mutations are in the N-terminus and included in the Yn, for Yc, we just use that of the EYFP (residues 156–239) because it was already made (Chen et al., 2007).

Ras proteins undergo important posttranslational modifications at the C-terminus to influence where they localize in the cell (Ahearn, Haigis, Bar-Sagi, & Philips, 2012). The C-terminus is also cleaved by a protease. We thus believe that it is better to tag Ras proteins at the N-terminus. As would be expected, in the BiFC system, the binding to the effector is also stronger with constitutively active (oncogenic) Ras proteins. We examined two of the most common oncogenic substitutions, G12V and Q61L, in H-Ras and found that the former binds slightly more strongly to the effectors. Seeking the appropriate negative controls for the BiFC system is critical and not trivial because when Yn and Yc are expressed at high enough levels in the same compartment, they too could interact to produce detectable fluorescence (Kodama & Hu, 2012). We created a Ras mutant as negative control, H-Ras E(G12V), in which all the amino acid residues in the effector-binding region (residues 33–40) of H-Ras(G12V) were mutated to alanines. This works much better than the N17 dominant-negative Ras proteins, which returns higher “background.” We note that even the binding to H-Ras E(G12V) is not “zero” with the effectors we have tested, and we have thus also examined GST (see in the succeeding text) as a negative control. To reduce background due to overexpression as a result of transient transfection, we have created cell lines in which fusion proteins are stably expressed at appropriate levels.

Before the screen, in addition to Cdc42, we have examined two known Ras effectors, Raf and RIN1, by BiFC as positive controls. While full-length Raf can readily bind H-Ras(G12V), a truncated form of Raf, Raf-RBD, containing just the Ras-binding domain, binds even more strongly to Ras. Thus, for general studies of Ras-effector interactions, Raf-RBD is an ideal positive control. As discussed in the preceding text, we believe that Ras proteins should be tagged at the N-terminus, and in the BiFC system, they were tagged with Yn. Conversely, Yc was fused at the C-terminus to the effectors. Since the authentic N- and C-termini of the YFP are maintained (and exposed) in the correct orientations in the fusion proteins, we postulate that the binding between Ras and the effector would not substantially interfere with the refolding of the YFP. We stress that we have not vigorously tested this hypothesis, and some of the isolated effectors (e.g., TESC) can bind Ras no matter whether they are N- and C-terminally tagged (Zheng, Cheng, et al., 2012). cDNAs expressing the described fusion proteins were carried by pBabe vectors, modified by us to make it easier for constructing BiFC fusion proteins (Chen et al., 2007).

Finally, a variety of cell lines have been used successfully for the BiFC experiments (Kerppola, 2006). We have chosen the human fibrosarcoma HT1080 cells in part due to their relatively low autofluorescence. For example, when examining Yn-H-Ras E(G12V) and Yc-Raf-RBD by FACS, only 0.6% of HT1080 cells showed detectable fluorescence, 20 × lower than that in MCF-7 cells. In addition, HT1080 cells endogenously overexpress an oncogenic N-Ras, which is at least partly responsible for the transforming phenotype in these cells (Zheng, Cheng, et al., 2012). As such, one can use the same cells to functionally validate the roles of novel Ras-binding proteins that act in the Ras pathways operative in these cells. One caveat with HT1080 is that they are prone to dying when transfected with Lipofectamine 2000.

2.2. Detect Ras-effector binding by FACS

1. Seed HT1080 cells in six-well plates and grow to 30–40% confluence.
2. Cotransfect 1 µg pBabe-Yc-Raf-RBD with 1 µg of either pBabe-Yn-H-Ras(G12V) or pBabe-Yn-H-Ras E(G12V) to each well using 6 µl Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA). pBabe-Yc-Raf-RBD and pBabe-Yn empty vectors were also cotransfected as an additional negative control.
3. Incubate these cells at 37 °C with CO₂ for overnight.
4. Trypsinize and pellet the cells by centrifugation at 150 × *g* at room temperature for 5 min.
5. Resuspend the cells in 300 µl of DMEM supplemented with 10% FBS and 4 µg/ml propidium iodide, which was used during FACS to exclude dead cells, and transfer them to 4 °C for 30 min.
6. Examine these cells by FACS for YFP. As shown in Fig. 2.1B, the YFP signal in Raf-RBD cells expressing H-Ras(G12V) can be readily separated from those carrying the negative controls.

2.3. Detect Ras–effector binding by microscopy

1. Seed HT1080 cells in 24-well glass bottom plates (MatTek), and grow them to 30–40% confluence.
2. Cotransfect 0.2 µg pBabe-Yc-Raf-RBD with 0.2 µg pBabe-Yn-H-Ras(G12V) or pBabe-Yn-H-Ras E(G12V) vector to each well using 1.2 µl Lipofectamine 2000.
3. Incubate these cells at 37 °C with CO₂ for overnight.
4. Replace the transfection media with phenol red-free DMEM supplemented with 2% FBS.
5. Examine the cells on a Leica TCS SP5 confocal microscope with a 63 ×/1.4 oil objective lens. The reconstituted YFP signals (Fig. 2.1C) were imaged as described previously (Zheng, Cheng, et al., 2012).

3. SCREEN FOR RAS-BINDING PROTEINS BY BiFC

3.1. Create “baits” and negative controls

We used Yn-H-Ras(G12V) as the bait. In addition to Yn-H-Ras E (G12V), we also used Yn-GST as a negative control, in case some effectors still retain a weak binding to H-Ras E(G12V). To reduce background and improve reproducibility, we found it helpful when the bait is stably expressed as follows:

1. Seed HEK293FT cells (less than 30 passages) in DMEM supplemented with 10% FBS in 10 cm Petri dishes. Grow the cells to 60–70% confluence.
2. Transfect 6 µg pBabe-Yn-H-Ras(G12V), pBabe-Yn-H-Ras E (G12V), or pBabe-Yn-GST plasmid individually with 6 µg retroviral packaging pAmpho vector into HEK293FT cells using 36 µl Lipofectamine 2000.
3. Incubate the cells in a 37 °C CO₂ incubator overnight.
4. Carefully aspirate the transfection medium, and replace it with 5 ml DMEM 10% FBS.
5. Incubate the cells in at 37 °C with CO₂ for 48 h.
6. Harvest the viruses by filtering the media through 0.45 µm PES syringe-top filter.
7. Mix the filtered virus-containing medium (~5 ml) with 2.5 ml fresh DMEM 10% FBS and 4 µg/ml polybrene and then added to the preseeded HT1080 cells (20–30% confluence) in 10 cm Petri dishes.
8. Incubate HT1080 cells at 37 °C with CO₂ for 36 h.
9. Replace the infection medium with DMEM 10% FBS and 500 µg/ml G418.
10. Incubate the cells at 37 °C with CO₂ for 2 weeks. Replace with fresh G418-containing growth medium every 3 days.
11. Pick single colonies and expand the cells.

12. Harvest the cells by a cell scraper with a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl₂, 10% glycerol, 1% NP-40, 1 mM PMSF, and a proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN).
13. Examine the expression level of Yn-H-Ras(G12V), Yn-H-Ras E (G12V), or Yn-GST in HT1080 clones by Western blots.
14. Select and pool three clones expressing medium levels of tested proteins.

3.2. Create cDNA libraries

We used human ORFeome v1.1 containing 8,100 ORFs as the resource of cDNAs (Rual et al., 2004). To rule out the possibility that the binding between Ras and some effectors may be sensitive to whether the effectors are tagged at the C- or N-terminus, we built two libraries, nYc and cYc (Zheng, Cheng, et al., 2012), in which the cDNAs were fused to the 5' and 3' of the coding sequence of Yc by the Gateway cloning system.

3.3. Initial screen for binding partners by FACS

1. Seed 5×10^5 of the “bait” cells (HT1080 cells stably expressing Yn-H-Ras(G12V)) to 10-cm Petri dishes; 24 h later, transfect 4 μ g library DNAs using 16 μ l Lipofectamine 2000.
2. For the negative control, transfect pBabe-Yc-Raf-RBD into HT1080-Yn-H-Ras E(G12V) cells. This can be done at a smaller scale since only a small amount of cells are needed for gating.
3. Incubate these cells at 37 °C with CO₂ for 24 h.
4. Trypsinize and pellet the cells by centrifugation at $150 \times g$ at room temperature for 5 min.
5. Resuspend the cells in DMEM supplemented with 10% FBS and 4 μ g/ml propidium iodide to a density of 1×10^6 cells/ml. Incubate at 4 °C for 30 min.
6. To set the gate, analyze the control cells (carrying the Yc-Raf-RBD/Yn-H-Ras E(G12V) pair) first for YFP signal by FACS. Collect all the cells carrying the cDNA that are outside the boundary set by the negative control. We note that this is intended to maximize the yield, and the other assays discussed in the succeeding text are designed to more stringently exclude false-positives.
7. Centrifuge the isolated cells at $150 \times g$ at room temperature for 5 min.
8. Resuspend the cell pellet with 300 μ l DNA extraction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, 1% SDS, and 0.2 mg/ml protease K.
9. Incubate the cells at 55 °C for 1 h.
10. Add 0.5 mg/ml RNase A to the cells, and incubate them at room temperature for another 1 h.
11. Extract the DNA by phenol/chloroform/isoamyl alcohol (25:24:1), and then precipitate the DNA by 1/10 volume of 3 N sodium acetate, 3 volume of ethanol,

and 50 µg/ml of glycogen. Wash with 70% ethanol, and finally, allow it to be air-dried at room temperature.

12. Dissolve the DNA in 10 µl TE buffer (pH 8.0), and transform the isolate DNAs into high-efficiency 10-beta bacterial cells (New England Biolabs, Ipswich, MA).
13. Pick colonies and extract the plasmid DNAs by QIAprep 96 Turbo Miniprep Kit (Qiagen).

3.4. Follow-up screens to confirm the binding

1. Seed 3×10^4 /well HT1080 cells stably expressing Yn-tagged H-Ras(G12V), H-Ras E(G12V), or GST into 24-well plates.
2. Grow the cells in at 37 °C with CO₂ for 24 h.
3. Transfect 0.2 µg of each isolated cDNA clone using 0.8 µl Lipofectamine 2000 to each well of cells. Transfect pBabe-Yc and pBabe-RIN1-Yc individually into all the three cell lines (Step 1, above) as the controls.
4. Incubate the cells in a 37 °C CO₂ incubator for overnight.
5. Trypsinize the cells, and pellet them by centrifugation at $150 \times g$ at room temperature for 5 min.
6. Resuspend the cells in 200 µl DMEM supplemented with 10% FBS and 4 µg/ml propidium iodide. Incubate at 4 °C for 30 min.
7. Examine the cells for YFP by FACS.
8. To readily exclude nonspecific binding, we estimated the binding strength between each protein pair by calculating the geometric mean of the reconstituted YFP intensity using FlowJo. We then divided the “binding” to the bait with that to either GST or H-Ras E(G12V) (see Supplemental figure S2 in Zheng, Cheng, et al. (2012), leading to the final selection of 64 cDNA clones with a relative binding that is >1 with *both* negative controls. To further exclude false positive clones in a more stringent manner, we performed two experiments to identify Ras-binding proteins that also affect Ras functions.

3.5. Functional validation

Oncogenic Ras proteins induce cellular transformation; we thus measured whether overexpressing the isolated cDNAs can alter the ability of NIH3T3 cells carrying H-Ras(G12V) to form colonies in soft agar (Zheng, Cheng, et al., 2012). In addition, we carried out an Elk1-mediated luciferase assay to measure gene expression as induced by the Ras-Raf-MAP kinase pathway. Since we conducted these studies using standard protocols, which are published, we will not describe them here in detail. We should point out, however, that out of the final 26 clones, only one can enhance these Ras activities, while the rest inhibit them. At first glance, this seems rather counterintuitive. In the case of CHMP6 and VPS4A, it is evident that overexpressing these two genes created dominant-negative effects on the Ras pathways; thus, we expect that overexpressing many other isolated cDNAs may similarly act in a dominant-negative fashion. Of course, another possibility is that some of

the isolated cDNAs control Ras activities that are different from those tested here. Regardless, it is critical to select Ras activities that are most relevant to your interest when designing such a screen. While the observed dominant-negativity associated with gene overexpression has caveats, it is convenient for narrowing down the list of potential Ras effectors. Ultimately, gene silencing is likely to be more informative in determining how isolated components work in a particular Ras pathway.

4. CONCLUDING REMARKS

While Ras proteins are well known to mediate growth factor signaling at the plasma membrane, we and others have discovered that Ras proteins can signal from endosomes. In particular, the results described in the preceding text strongly suggest that Ras proteins can activate the ESCRIT-III complex to induce recycling of components of the Ras pathway to enhance growth factor signaling. Besides Cdc42, CHMP6, and VPS4A, PI3K, one of the most cancer-relevant Ras effectors, has also been shown to interact with Ras proteins in the endosome (Tsutsumi, Fujioka, Tsuda, Kawaguchi, & Ohba, 2009). Therefore, better understanding of the roles of Ras in endosomes is likely to uncover key mechanisms by which Ras proteins are involved in tumorigenesis.

It is perhaps not surprising that Ras proteins move dynamically in the cell and along the way interact with many effectors. BiFC has offered us a great tool to identify these interacting proteins because of its unique property to secure protein–protein interactions that are otherwise transient and weak. Despite this, in reflection, the BiFC system can be improved in several important ways with improved reagents and technology.

The single most labor-intensive (and costly) step in our screen was to sort out individual cDNA clones from the pool. We have first tried to sort out individual YFP⁺ cells but found that only a very few cells survived the sorting, and they frequently carrying the cDNAs expressing Raf. Thus, this approach may work if your genes of interest promote cell survival after sorting. We have also sought to use the pooled isolated cDNAs to conduct a second round of screening using FACS with the intention to “enrich” positive clones. However, we found that some positive clones, such as A-Raf, that were present in the initial pool were in fact lost in the second screen. We speculate that the BiFC signals are greatly influenced by copy number of the vector, which is hard to control during transfection. This problem can be addressed using cDNA libraries that are printed in 96-well format and by screening through a robotic automated FACS system. Finally, more Ras effectors can be found if the BiFC libraries have better coverage of the human genome. We note that since the completion of our study, over 90% of human cDNAs are now available (Team et al., 2009).

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Figure 2.1.

Detecting H-Ras and Raf-RBD interaction by BiFC. (A) A schematic representation of the BiFC system—see text for details. (B) Yc-tagged Raf-RBD was cotransfected with indicated Yn-tagged constructs into HT1080 cells. The transfected cells were cultured for another 36 h before being examined by FACS analysis for YFP. (C) The same cells as in (B) were examined by confocal microscopy for the reconstituted YFP signal. Cells were also stained with Hoechst 33258 to mark nuclei. Cells expressing the Yn control yielded the same results as those with H-Ras(G12V) E (not shown).