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RESEARCH ARTICLE

Dimerization drives EGFR endocytosis through two sets of compatible endocytic codes

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ABSTRACT

We have shown previously that epidermal growth factor (EGF) receptor (EGFR) endocytosis is controlled by EGFR dimerization. However, it is not clear how the dimerization drives receptor internalization. We propose that EGFR endocytosis is driven by dimerization, bringing two sets of endocytic codes, one contained in each receptor monomer, in close proximity. Here, we tested this hypothesis by generating specific homo- or hetero-dimers of various receptors and their mutants. We show that ErbB2 and ErbB3 homodimers are endocytosis deficient owing to the lack of endocytic codes. Interestingly, EGFR–ErbB2 or EGFR–ErbB3 heterodimers are also endocytosis deficient. Moreover, the heterodimer of EGFR and the endocytosis-deficient mutant EGFRΔ1005–1017 is also impaired in endocytosis. These results indicate that two sets of endocytic codes are required for receptor endocytosis. We found that an EGFR–PDGFRβ heterodimer is endocytosis deficient, although both EGFR and PDGFRβ homodimers are endocytosis-competent, indicating that two compatible sets of endocytic codes are required. Finally, we found that to mediate the endocytosis of the receptor dimer, the two sets of compatible endocytic codes, one contained in each receptor molecule, have to be spatially coordinated.

KEY WORDS: EGF receptor, Endocytosis, Dimerization, Dual endocytic codes

INTRODUCTION

The epidermal growth factor (EGF) receptor (EGFR), also known as HER1 and ErbB1, is the prototypal member of the superfamily of receptors with intrinsic tyrosine kinase activity and is widely expressed in many cell types, including those of epithelial and mesenchymal lineages (Wells, 1999; Yarden and Sliwkowski, 2001). The other three members of the ErbB receptor family include Her2/ErbB2/neu (Bargmann et al., 1986; Yamamoto et al., 1986), Her3/ErbB3 (Kraus et al., 1989) and Her4/ErbB4 (Plowman et al., 1993). EGFR is a 170-kDa membrane glycoprotein composed of three domains. The heavily glycosylated 622-amino-acid extracellular domain is responsible for ligand binding and receptor dimerization. The transmembrane domain is a single 23-residue α-helical transmembrane peptide. The 542-residue intracellular cytoplasmic domain contains a 250-amino-acid conserved protein tyrosine kinase core followed by a 229-residue C-terminal tail with regulatory tyrosine residues. The EGFR family of receptor tyrosine kinases initiates a complex signal transduction cascade that modulates cell proliferation, survival, adhesion, migration and differentiation (Wells, 1999; Yarden and Sliwkowski, 2001; Citri and Yarden, 2006). Although growth factor-induced EGFR signaling is essential for many normal morphogenic processes and is involved in numerous additional cellular responses, the aberrant activity of members of this receptor family has been shown to play a key role in the development and growth of tumor cells (Citri and Yarden, 2006; Burgess, 2008; Hynes and MacDonald, 2009).

The endocytosis of EGFR is initiated by binding to EGF. The events induced by EGF-binding likely regulate EGFR–EGFR endocytosis. These events include receptor dimerization, activation of intrinsic tyrosine kinase activity and autophosphorylation. We have shown that EGFR internalization is controlled by EGFR dimerization, rather than EGFR kinase activation, and the EGFR C-terminal sequence of amino acids 1005–1017 and dileucine motif $L^{1010}L^{1011}$ function as endocytic codes to mediate dimerization-driven EGFR endocytosis, independent of receptor kinase activity (Wang et al., 2005; Wang et al., 2007). However, it is not known how the dimerization drives the receptor internalization.

It has been shown that the other members of the ErbB receptor family are impaired in endocytosis by using EGF-responsive chimeric receptors (Sorkin and Carpenter, 1993; Baulida et al., 1996; Wang et al., 1999). It has also been indicated that the endocytosis-inefficient ErbB2 and ErbB3 are often recycled back to the cell surface (Pinkas-Kramarski et al., 1996; Lenferink et al., 1998; Waterman et al., 1998). The poor binding of Cbl to ErbB2, ErbB3 or the EGFR–ErbB2 heterodimer might contribute to the recycling (Levkowitz et al., 1996; Muthuswamy et al., 1999). However, ErbB2 has been reported to be capable of internalization upon binding with certain monoclonal antibodies (mAbs), and that mAb-induced ErbB2 endocytosis is strictly dependent on antibody bivalency (Drebin et al., 1985; Gilboa et al., 1995; Klapper et al., 1997). These results suggest that, like EGFR homodimers, ErbB2 homodimers are capable of endocytosis. It has been shown that EGFR can form both homodimers and heterodimers with ErbB2 following EGF stimulation (Yarden and Schlessinger, 1987; Goldman et al., 1990; Carraway and Cantley, 1994; Qian et al., 1994;). However, the EGFR–ErbB2 heterodimer was impaired in endocytosis (Wang et al., 1999; Worthylake et al., 1999; Shankaran et al., 2006). These data indicate that receptor homodimerization rather than heterodimerization might improve EGFR internalization, but it is still not clear what is the underlying mechanism. The different conformational changes induced by homodimerization or heterodimerization might account for this difference.

In this study, we exploit a controllable system to specifically examine the endocytosis of receptor homodimers and

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heterodimers. We show that ErbB2 and ErbB3 homodimers are endocytosis deficient owing to the lack of endocytic codes in their C-termini. Interestingly, EGFR–ErbB2 or EGFR–ErbB3 heterodimers are also endocytosis deficient. Moreover, the heterodimer between EGFR and the endocytosis-deficient mutant EGFRΔ1005–1017 is also impaired in endocytosis despite the presence of endocytic codes in EGFR. These results indicate that two endocytic codes are required for receptor endocytosis. We further show that the EGFR and PDGFRβ heterodimer is endocytosis deficient, although both EGFR and PDGFRβ homodimers are endocytosis competent, which indicates that two compatible endocytic codes are required. Finally, we show that to mediate the endocytosis of the receptor dimer, the two compatible endocytic codes, one contained in each receptor molecule, have to be spatially coordinated.

RESULTS

Receptor chimeras and controlled homo- and hetero-dimerization

To develop a controllable homo- and hetero-dimerization system to specifically study the internalization of receptor homodimers or heterodimers, we utilized the ARGENT^{TM} Regulated Homodimerization and ARGENT^{TM} Regulated Heterodimerization receptor dimerization kits. In this system, chemicals can be used to specifically homodimerize two proteins fused with FK506-binding protein (FKBP) or heterodimerize two proteins fused with FKBP and the FKBP12-rapamycin-binding (FRB) domain from FKBP-12-rapamycin associated protein 1, respectively (Muthuswamy et al., 1999; Zhan et al., 2006) (supplementary material Fig. S1). In this study, we constructed a series of receptor chimeras by fusing FKBP or FRB to the C-terminus of these receptors. Following transfection of these chimeras into 293T cells, stimulation with the homodimerizer AP20187, can induce homodimerization between receptors fused with FKBP, and stimulation with the heterodimerizer AP21967, can induce heterodimerization between the FKBP-fused receptor and FRB-fused receptor (supplementary material Fig. S1). All of the FKBP fusion receptors were also epitope-tagged with Myc, and the FRB fusion receptor was epitope-tagged with HA to allow specific detection with antibodies.

Endocytosis of ErbB family receptor homodimers

We have previously shown that similar to EGF, AP20187 induced the homodimerization of EGFR–FKBP, which led to the activation and internalization of EGFR–FKBP (Wang et al., 2005). To determine whether homodimerization also drives the internalization of ErbB2 or ErbB3, we fused FKBP with both ErbB2 (ErbB2–FKBP) and ErbB3 (ErbB3–FKBP) (Fig. 1A). We then transfected 293T cells with EGFR–FKBP, ErbB2–FKBP or ErbB3–FKBP, and examined the effect of AP20187 on receptor dimerization, activation and endocytosis. The dimerization of the receptors was determined by a cross-linking assay. As shown in Fig. 2A, following AP20187 stimulation for 30 min, EGFR–FKBP, ErbB2–FKBP and ErbB3–FKBP all formed homodimers. EGF and heregulin (HRG, encoded by *NRG1*) also stimulated the homodimerization of EGFR–FKBP and ErbB3–FKBP, respectively (Fig. 2A). We then examined the endocytosis of these receptor homodimers by following a cell surface biotinylation signal. As shown in Fig. 2B,C, whereas EGFR–FKBP was strongly internalized following addition of EGF or AP20187 for 5 to 30 min, both ErbB2–FKBP and ErbB3–FKBP were not endocytosed following AP20187 addition for 5, 15 and 30 min. Moreover, whereas HRG had induced homodimerization of ErbB3–FKBP (Fig. 2A), it did not stimulate the endocytosis of ErbB3–FKBP, either (Fig. 2B,C). Indirect immunofluorescence was conducted to further examine the endocytosis of these receptor homodimers. As shown in Fig. 2D,E, incubation with AP20187 did not stimulate the endocytosis of either ErbB2 or ErbB3, which is consistent with previous reports which have shown that ErbB2 and ErbB3 homodimers are internalization deficient (Sorkin and Carpenter, 1993; Baulida et al., 1996; Wang et al., 1999). We also showed that AP20187 induced the phosphorylation of ErbB2 (Fig. 2D), which is consistent with previous reports showing that overexpressed ErbB2 is readily available for phosphorylation owing to its constitutively exposed dimerization domain (Schlessinger, 2002; Garrett et al., 2003;). However, ErbB3 was not phosphorylated upon stimulation with either AP20187 or its physiological ligand HRG owing to its lack of intrinsic kinase activity (Fig. 2D). As a control, we have shown here that both EGF and AP20187 induced the phosphorylation and endocytosis of EGFR in 293T cells transfected with EGFR–FKBP (Fig. 2D,E). Taken together, these data indicate that ErbB2 and ErbB3 homodimers are endocytosis deficient, and this deficiency might be due to a lack of endocytic codes in the C-terminal regulatory domains of the receptors.

Impaired endocytosis of EGFR–ErbB2 and EGFR–ErbB3 heterodimers

We have shown that EGFR internalization is controlled by EGFR dimerization (Wang et al., 2005). We propose that the underlying mechanism for this internalization is that two sets of endocytic codes, one contained in each receptor molecule of the dimer, are required for mediating EGFR internalization. We have shown above and previously that the endocytic codes contained in the EGFR C-terminus mediate the endocytosis of the EGFR homodimer (Wang et al., 2005; Wang et al., 2007). By contrast, the impaired endocytosis of ErbB2 and ErbB3 homodimers as shown above suggests that both ErbB2 and ErbB3 lack the endocytic codes. Thus, the heterodimers of EGFR–ErbB2 and EGFR–ErbB3 serve as good models to test our hypothesis. To examine the endocytosis of EGFR–ErbB2 and EGFR/ErbB3 heterodimers, we fused FRB with EGFR (EGFR–FRB) (Fig. 1A) and then transfected 293T cells with EGFR–FRB and ErbB2–FKBP or ErbB3–FKBP. The cells were then stimulated with EGF, HRG or AP21967. The formation of receptor heterodimers was determined by co-immunoprecipitation experiments followed by analysis by immunoblot. We showed that in 293T cells co-transfected with EGFR–FRB and ErbB2–FKBP, both EGF and AP21967 induced a strong association between EGFR–FRB and ErbB2–FKBP (Fig. 3A). However, as shown by following the cell surface biotinylation signal, AP21967 did not stimulate the endocytosis of either EGFR–FRB or ErbB2–FKBP (Fig. 3B,C). Given that AP21967 specifically induces the formation of the (EGFR–FRB)–(ErbB2–FKBP) heterodimers, without the formation of the EGFR–FRB homodimers or ErbB2–FKBP homodimers, our results indicated that EGFR–ErbB2 heterodimers are endocytosis deficient.

Similarly, for 293T cells co-transfected with EGFR–FRB and ErbB3–FKBP, both HRG and AP21967 stimulated a strong association between EGFR–FRB and ErbB3–FKBP (Fig. 3D). However, AP21967 did not stimulate the endocytosis of either EGFR–FRB or ErbB3-FKBP as shown following the cell surface biotinylation signal (Fig. 3E,F), which indicates that EGFR–ErbB3 heterodimers are also endocytosis deficient.

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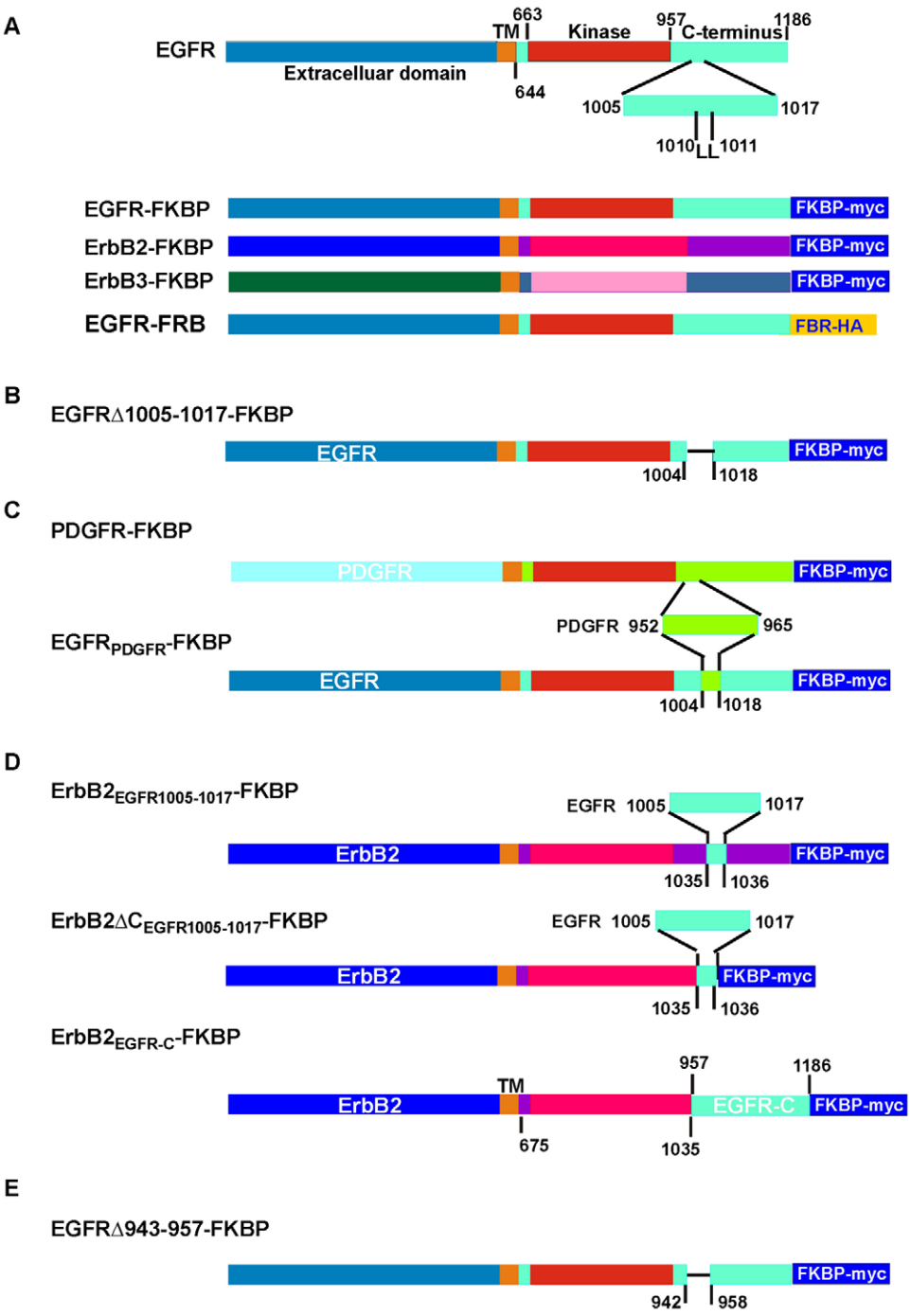

Fig. 1. Schematic representation of various receptors and mutants constructed and used in this research. All of the FKBP fusion receptors were also epitope-tagged with HA and the FRB fusion receptor was epitope tagged with Myc. TM, transmembrane domain.

The endocytosis of EGFR–ErbB2 and EGFR–ErbB3 heterodimers was further examined by indirect immunofluorescence. As shown in Fig. 3G,H, AP21967 did not stimulate the endocytosis of EGFR–FRB, ErbB2–FKBP and ErbB3–FKBP. Our results confirmed that both EGFR–ErbB2 and EGFR–ErbB3 heterodimers are endocytosis deficient. Given that the EGFR molecule within the heterodimer contains the necessary endocytic codes for internalization, these results suggest that two sets of endocytic codes are required to mediate receptor endocytosis, or alternatively, the inhibitory effect of the ErbB2 and ErbB3 C-terminus caused the endocytosis deficiency of EGFR–ErbB2 or EGFR–ErbB3 heterodimers.

It is interesting to note that for 293T cells co-expressing EGFR–FRB and ErbB2–FKBP, the addition of EGF did not stimulate the internalization of ErbB2–FKBP, but stimulated some EGFR internalization (Fig. 3B,C,G,H). It is well established that EGF can induce the formation of both EGFR homodimers and EGFR–ErbB2 heterodimers, and heterodimer formation is preferred in cells co-expressing EGFR and ErbB2 (Goldman et al., 1990; Qian et al., 1994; Soltoff et al., 1994). Given that the EGFR homodimer is efficiently internalized following EGF-induced dimerization, it is likely that the observed EGFR endocytosis is due to the formation of EGFR homodimer. The reduced EGFR endocytosis in response to EGF is also an

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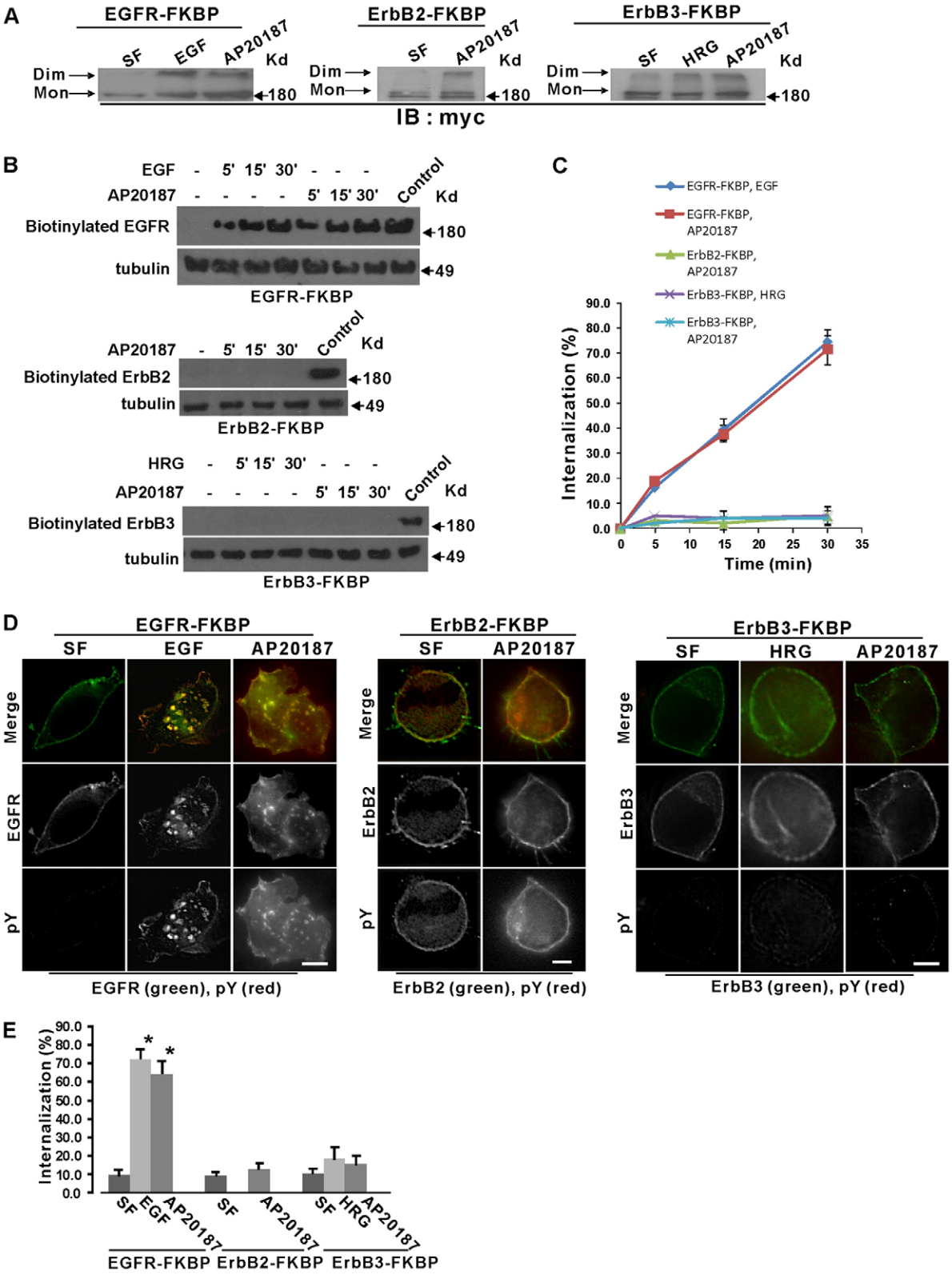

Fig. 2. See next page for legend.

indication that the EGFR–ErbB2 heterodimer is impaired in internalization. By contrast, for 293T cells expressing both EGFR–FRB and ErbB3–FKBP, the addition of HRG did not stimulate any endocytosis of EGFR and ErbB3 (Fig. 3D–H). Given that HRG only stimulates the homodimerization of ErbB3, but not EGFR, this result is consistent with the findings that both ErbB3 homodimers and EGFR–ErbB3 heterodimers are impaired in endocytosis.

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Fig. 2. Dimerization and endocytosis of ErbB family receptors. 293T cells transiently transfected with EGFR–FKBP, ErbB2–FKBP or ErbB3–FKBP were treated with EGF (50 ng/ml), AP20187 (100 nM), or HRG (50 ng/ml) for 30 min or as indicated. SF, serum free; HRG, heregulin; Dim, dimer; Mon, monomer. (A) Dimerization. The proteins were crosslinked with disuccinimidyl suberate (DSS), and then the cells were lysed and the protein samples were immunoblotted with anti-Myc antibody. (B) Endocytosis of ErbB receptors following homodimerization by cell surface biotinylation. The endocytosis of ErbB receptors was examined by following the cell surface biotinylation signal as described in the Materials and Methods. Control, total biotinylated receptors without incubation with 100 mM MESNA at 4°C for 10 min to remove biotin from the sulfo-NHS-SS-biotin-labeled proteins on the cell surface and without incubation with ligands or dimerizers. (C) Quantification of the data from B. Each value is the mean±s.e.m. of at least three experiments. (D) Endocytosis of ErbB receptors following homodimerization determined by indirect immunofluorescence. The localization of EGFR–FKBP, ErbB2–FKBP or ErbB3–FKBP (green) and phosphotyrosine (pY; red) were analyzed by double indirect immunofluorescence as described in the Materials and Methods. Scale bars: 20 μm. (E) Quantification of the data from D as described in the Materials and Methods. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF.

Two sets of endocytic codes are required for the endocytosis of EGFR dimers

The impaired endocytosis of the EGFR–ErbB2 and EGFR–ErbB3 heterodimers might be due to either the lack of an endocytic code in ErbB2 and ErbB3, or the presence of endocytosis-inhibitory signals in ErbB2 and ErbB3. To further examine whether two sets of endocytic codes in the EGFR dimer are required to mediate its internalization, we checked the endocytosis of EGFR heterodimers between wild-type EGFR and an EGFR mutant lacking endocytic codes. We have previously shown that the C-terminus regulatory domain of EGFR, especially amino acid residues 1005–1017 and the di-leucine motif $L^{1010}L^{1011}$, are essential for EGFR endocytosis (Wang et al., 2007). We fused EGFRΔ1005–1017 with FKBP (EGFRΔ1005–1017–FKBP) (Fig. 1B). Then, we expressed EGFRΔ1005–1017–FKBP and the above described EGFR–FRB in 293T cells and stimulated the cells with either EGF or AP21967. The co-immunoprecipitation assays showed that both EGF and AP21967 induced the association of these two receptors (Fig. 3I). As shown by following the cell surface biotinylation signal, upon EGF stimulation, we did not observe the internalization of EGFRΔ1005–1017–FKBP. The internalization of EGFR–FRB was greatly reduced, but not eliminated (Fig. 3J,K), which might be due to the formation of some EGFR–FRB homodimers. However, when treated with AP21967, no detectable internalization of either EGFR–FRB or EGFRΔ1005–1017–FKBP was observed (Fig. 3J,K). These results indicate that the heterodimer of EGFR–FRB and the EGFRΔ1005–1017 mutant is impaired in endocytosis.

We further analyzed the endocytosis of the (EGFR–FRB)–(EGFRΔ1005–1017) dimer by indirect immunofluorescence. Consistent with our biotinylation results, upon EGF stimulation, we did not observe the internalization of EGFRΔ1005–1017–FKBP. The endocytosis of EGFR–FRB was greatly reduced, but not eliminated (Fig. 3L,M). However, when treated with AP21967, no detectable internalization of either EGFR–FRB or EGFRΔ1005–1017–FKBP was observed (Fig. 3L,M). These results further indicate that the heterodimer of EGFR–FRB and the EGFRΔ1005–1017 mutant is impaired in endocytosis. Given that the EGFRΔ1005–1017 mutant does not contain an inhibitory signal, but only lacks the endocytic codes in its C-terminus, our results indicate that two sets of endocytic codes are required to mediate EGFR internalization.

Two non-compatible endocytic codes failed to induce EGFR internalization

We next examined whether the two sets of endocytic codes need to be identical or compatible in order to mediate EGFR endocytosis. In other words, are any two sets of endocytic codes sufficient for receptor endocytosis. To test this, we created a receptor dimer containing two sets of functional, but different, endocytic codes. We have shown that homodimerization of platelet-derived growth factor receptor β (PDGFRβ) is necessary and sufficient to stimulate PDGFRβ endocytosis (Pahara et al., 2010). Thus, a heterodimer of EGFR and PDGFRβ would serve as a good model to test this notion. We fused PDGFRβ with FKBP (PDGFRβ–FKBP) (Fig. 1C) and expressed both PDGFRβ–FKBP and EGFR–FRB in 293T cells. We showed by co-immunoprecipitation that EGFR–FRB and PDGFRβ–FKBP formed a heterodimer following the treatment of the cells with AP20187 (Fig. 4A). We also found that the EGFR–PDGFRβ heterodimer was not internalized, as shown by both analysis of cell surface biotinylation (Fig. 4B,C) and immunofluorescence microscopy (Fig. 4D,E). As both EGFR and PDGFRβ contain endocytic codes capable of mediating the endocytosis of EGFR and PDGFRβ homodimers, respectively, our data suggest that the endocytic codes of EGFR and PDGFRβ are not compatible and two non-compatible endocytic codes are insufficient to mediate the endocytosis of receptor dimers.

To further test whether two compatible endocytic codes are required for mediating the endocytosis of receptor dimers, we replaced the EGFR endocytic code with the PDGFRβ endocytic code in the EGFR–FKBP receptor. Given that the hydrophobic region of amino acid residues 952–965 of the PDGFRβ C-terminus is crucial for PDGFRβ internalization (Mori et al., 1991; Pahara et al., 2010), we constructed a FKBP–fusion EGFR and PDGFRβ swap mutant with the replacement of the EGFR 1004–1017 residue hydrophobic region with the 952–965 residue fragment of PDGFRβ ($EGFR_{PDGFR}$–FKBP) (Fig. 1C). We then tested whether this mutant $EGFR_{PDGFR}$–FKBP was able to undergo endocytosis following homodimerization. As shown by following the cell surface biotinylation signal, upon both EGF and AP20187 treatment for 5–30 min, $EGFR_{PDGFR}$–FKBP was strongly internalized (Fig. 5A,B). Treatment with the EGFR kinase inhibitor AG1478 did not block the endocytosis of $EGFR_{PDGFR}$–FKBP (Fig. 5A,B). Similarly, we showed by indirect immunofluorescence that upon both EGF and AP20187 treatment for 15 min, $EGFR_{PDGFR}$–FKBP was strongly internalized with or without inhibition of its kinase activity by AG1478 treatment (Fig. 5C,D). These results indicate that the PDGFRβ endocytic code (residues 952–965) can also function as an endocytic codes for EGFR homodimers.

Now, we were able to analyze the endocytosis of the heterodimer of EGFR–FRB and $EGFR_{PDGFR}$–FKBP. We co-expressed both EGFR–FRB and $EGFR_{PDGFR}$–FKB in 293T cells and then treated the cells with AP21967. As shown in Fig. 5E, AP21967 stimulated the heterodimerization between EGFR–FRB and $EGFR_{PDGFR}$–FKBP, however, the EGFR–FRB and $EGFR_{PDGFR}$–FKBP heterodimer was not internalized, as shown by both by following the cell surface biotinylation signal (Fig. 5F,G) and by indirect immunofluorescence (Fig. 5H,I). Given that EGFR–FRB and $EGFR_{PDGFR}$–FKBP are both capable of endocytosis and only differ in endocytic codes, our results

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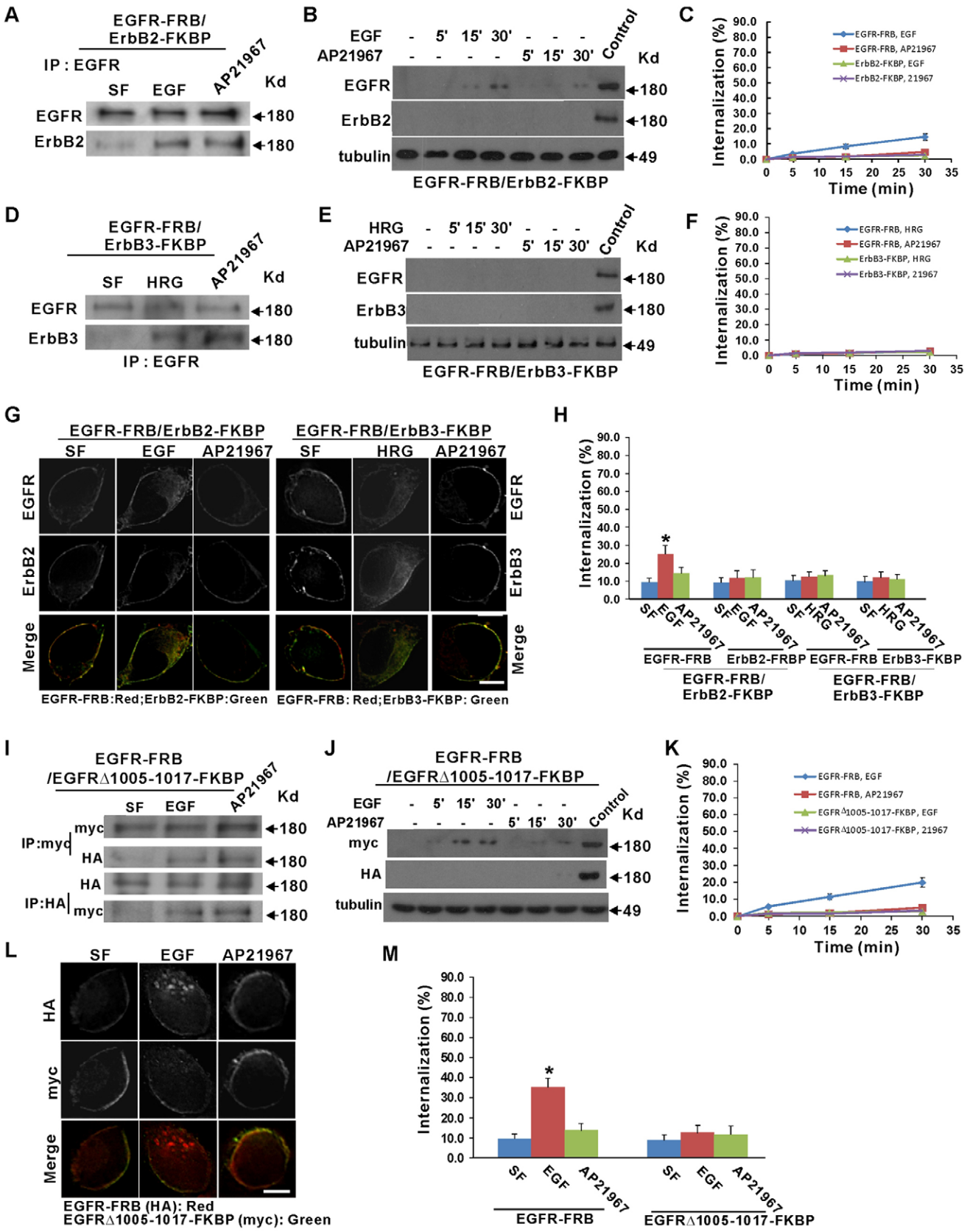

Fig. 3. See next page for legend.

Fig. 3. Heterodimerization and endocytosis of ErbB family receptors and mutants. (A–H) Heterodimerization and endocytosis of ErbB family receptors. 293T cells transiently co-transfected with EGFR–FRB and ErbB2–FKBP or ErbB3–FKBP were treated with EGF (50 ng/ml), HRG (50 ng/ml) or AP21967 (500 nM) for 30 min or as indicated. SF, serum free. (A) Heterodimerization of EGFR and ErbB2. The association (dimerization) between EGFR and ErbB2 was examined by co-immunoprecipitation (IP) as described in the Materials and Methods. (B) Endocytosis of the EGFR–ErbB2 heterodimer examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (C) Quantification of the data from B. Each value is the mean±s.e.m. of at least three experiments and the error bar is the standard error. (D) Heterodimerization of EGFR and ErbB3. The association (dimerization) between EGFR and ErbB3 was examined by co-immunoprecipitation. (E) Endocytosis of the EGFR–ErbB3 heterodimer examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (F) Quantification of the data from E. Each value is the mean±s.e.m. of at least three experiments. (G) Endocytosis of EGFR–ErbB2 and EGFR–ErbB3 heterodimers as determined by indirect immunofluorescence. The endocytosis of EGFR (red) and ErbB2 (green) or ErbB3 (green) was examined by double indirect immunofluorescence. Scale bar: 20 μm. (H) Quantification of data from G. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF. (I–M) Endocytosis of the EGFR–FRB and EGFRΔ1005–1017–FKBP heterodimers. 293T cells were transiently co-transfected with EGFR–FRB and EGFRΔ1005–1017–FKBP. The cells were then treated with EGF (50 ng/ml) or AP21967 (500 nM) for 30 min or as indicated. (I) Heterodimerization of EGFR–FRB and EGFRΔ1005–1017-FKBP. The association (dimerization) between EGFR–FRB and EGFRΔ1005–1017-FKBP was examined by co-immunoprecipitation. (J) Endocytosis of heterodimer of EGFR and EGFRΔ1005–1017 by cell surface biotinylation. Control is as described in the legend for Fig. 2B. (K) Quantification of the data from J. Each value is the mean±s.e.m. of at least three experiments. (L) Endocytosis of EGFR–FRB (red) and EGFRΔ1005–1017–FKBP (green) heterodimers was examined by double indirect immunofluorescence. Scale bar: 20 μm. (M) Quantification of data from L. Each value is the mean±s.e.m. of at least 8 cells from three different experiments. *$P<0.01$ when compared with SF.

suggest that two sets of non-compatible endocytic codes are unable to mediate the internalization of the receptor dimer. It is interesting to note that the AP21967 stimulated the phosphorylation of the heterodimer (Fig. 5E), which indicates that kinase activation is not sufficient to stimulate the endocytosis.

Multiple internalization signals are required to drive the internalization in a dimeric manner

Considering that the internalization deficiency of ErbB2 might be due to the lack of endocytic codes, we inserted EGFR residues 1005–1017 into the corresponding region of ErbB2 to generate an ErbB2–FKBP fusion mutant (ErbB2$_{EGFR1005–1017}$-FKBP) (Fig. 1D), and examined the internalization of ErbB2$_{EGFR1005–1017}$–FKBP. As shown in Fig. 6A, when expressed in 293T cells by transient transfection, ErbB2$_{EGFR1005–1017}$–FKBP was homodimerized and phosphorylated following AP20187 treatment. A low level of internalization of ErbB2$_{EGFR1005–1017}$–FKBP was also observed by both following the cell surface biotinylation signal (Fig. 6B,C) and by indirect immunofluorescence, which suggests that the endocytic codes within the EGFR fragment of residues 1005–1017 can partially restore the internalization of the ErbB2 homodimer. The lack of full restoration might be due to either the lack of other endocytic codes or the presence of an inhibitory sequence in ErbB2. To exclude the possibility that there are inhibitory signals in the C-terminus of ErbB2, we replaced the entire ErbB2 C-terminus with the EGFR endocytic code amino acid sequence 1005–1017 to create a mutant receptor ErbB2ΔC$_{EGFR1005–1017}$–FKBP (Fig. 1D). As shown in Fig. 6F, this mutant receptor was dimerized upon AP20187 treatment, but not phosphorylated. Moreover, this mutant was completely impaired in endocytosis following AP20187-induced homodimerization, as shown by both following the cell surface biotinylation signal (Fig. 6G,H) and by indirect immunofluorescence (Fig. 6I,J). These data suggest that other endocytic signals beyond the amino acid sequence 1005–1017 are also required to efficiently drive the internalization of ErbB receptors after dimerization. To confirm this possibility, we replaced the entire ErbB2 C-terminus with the EGFR C-terminus to create a hybrid receptor ErbB2$_{EGFR-C}$–FKBP (Fig. 1D). We transfected this hybrid receptor into 293T cells and examined its endocytosis. The addition of AP20187 induced the homodimerization of ErbB2$_{EGFR-C}$–FKBP (Fig. 6K), but unlike ErbB2ΔC$_{EGFR1005–1017}$–FKBP (Fig. 6F), this was followed by phosphorylation of ErbB2$_{EGFR-C}$–FKBP (Fig. 6K). Moreover, as shown by following the cell surface biotinylation signal, treatment with AP21087 for 5–30 min strongly stimulated the endocytosis of ErbB2$_{EGFR-C}$–FKBP (Fig. 6L,M). Similar results were obtained by indirect immunofluorescence (Fig. 6N,O) Therefore, we concluded that multiple endocytic codes, including residues 1005–1017 and other signals within the EGFR C-terminus, mediate EGFR internalization in a dimeric pattern. These results also confirmed that the endocytosis deficiency of ErbB2 results from the lack of endocytic codes in its C-terminus.

Two sets of compatible endocytic codes need to be spatially coordinated to mediate dimer internalization

Next, we determined whether additional conditions are needed for two sets of compatible endocytic codes to mediate the internalization of the ErbB receptors. We examined the internalization of (EGFR–FRB)–(ErbB2$_{EGFR1005–1017}$–FKBP) heterodimers. The (EGFR–FRB)–(ErbB2$_{EGFR1005–1017}$–FKBP) heterodimer contains a pair of identical endocytic codes (EGFR amino acid sequence 1005–1017), one code in each molecule. As shown in Fig. 7A, when co-expressed in 293T cells, EGFR–FRB and ErbB2$_{EGFR1005–1017}$–FKBP heterodimerized and were phosphorylated upon AP21967 treatment. This heterodimer, however, is endocytosis deficient, as shown by both by following the cell surface biotinylation signal (Fig. 7B,C) and indirect immunofluorescence (Fig. 7D,E). These results indicate that even the presence of a pair of identical endocytic signals might not be sufficient for receptor endocytosis. To confirm this notion, we examined the endocytosis of the (EGFR–FRB)–(ErbB2$_{EGFR-C}$–FKBP) heterodimer that contains two sets of identical and complete EGFR endocytic codes (the entire EGFR C-terminus), one set in each molecule. As shown in Fig. 7F, when co-expressed in 293T cells, EGFR–FRB and ErbB2$_{EGFR-C}$–FKBP heterodimerized and were phosphorylated upon AP21967 treatment. Interestingly, as shown by both by following the cell surface biotinylation signal (Fig. 7G,H) and indirect immunofluorescence (Fig. 7I,J), this heterodimer is endocytosis deficient, despite both the EGFR–FRB homodimer and the ErbB2$_{EGFR-C}$–FKBP homodimer being robustly endocytosed following the dimerization (Fig. 6K–O), and both containing the identical endocytic codes. One possible explanation for this impaired endocytosis is that spatial coordination of the two sets of compatible endocytic codes is required. As shown in Fig. 1, the EGFR C-terminus includes the amino acid sequence from 957 to 1186. When we constructed ErbB2$_{EGFR-C}$, we put the EGFR C-terminus after residue 1035 in ErbB2. The total amino acids between the end of transmembrane domain and the start of EGFR C-terminus is 313 (from 644–957) for EGFR and 360

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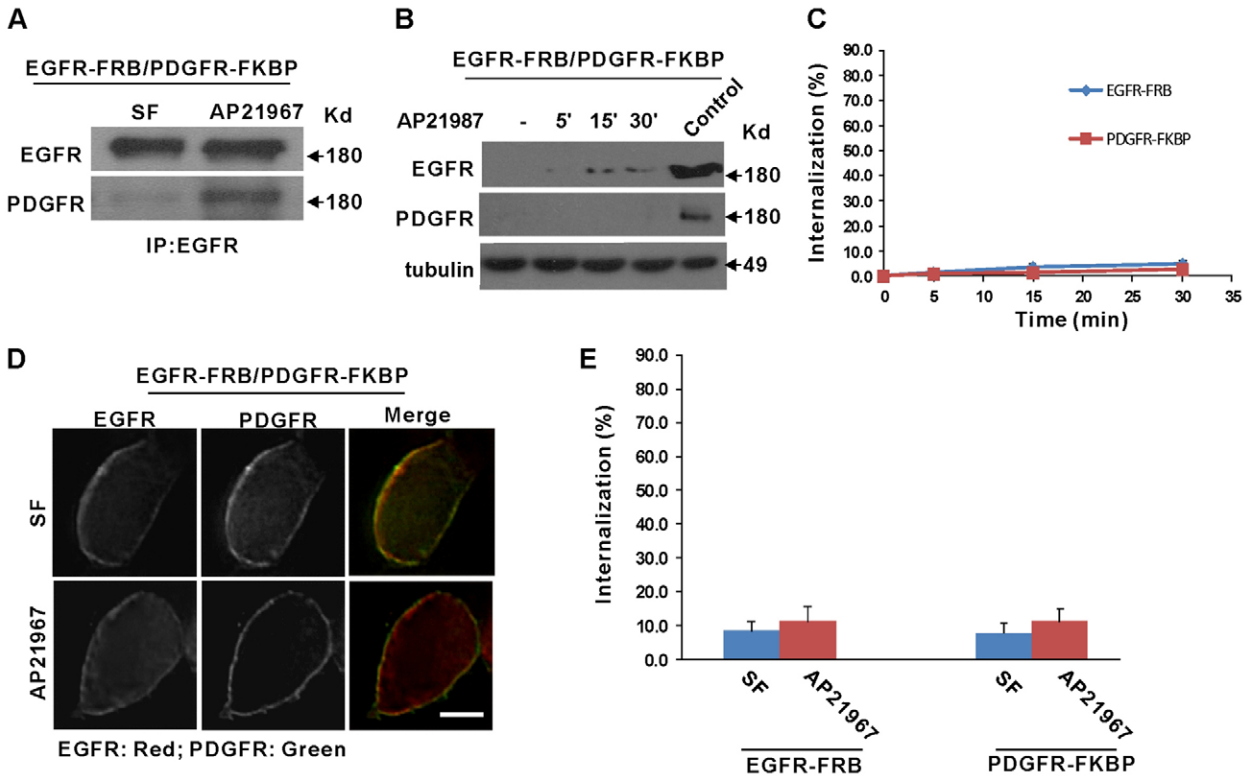

Fig. 4. Endocytosis of the EGFR–FRB and PDGFRβ–FKBP heterodimers. 293T cells were transiently co-transfected with EGF–FRB and PDGFRβ–FKBP. The cells were then treated with AP21967 (500 nM) for 30 min or as indicated. SF, serum free. (A) Heterodimerization of EGF–FRB and PDGFRβ–FKBP. The association (dimerization) between EGFR–FRB and EGFRΔ1005–1017–FKBP was examined by co-immunoprecipitation. (B) Endocytosis of EGF-FRB and PDGFRβ-FKBP heterodimers was examined by following the cell surface biotinylation signal. Control is as described in the Fig. 2B legend. (C) Quantification of the data from B. Each value is the mean±s.e.m. of at least three experiments. (D) Endocytosis of EGF–FRB (red) and PDGFRβ–FKBP (green) heterodimers examined by double indirect immunofluorescence. Scale bar: 20 μm. (E) Quantification of data from D. Each value is the mean±s.e.m. of at least eight cells from three different experiments.

(from 675–1035) for $ErbB2_{EGFR\text{-}C}$. Thus, the EGFR C-terminus that contains the endocytic codes within $ErbB2_{EGFR\text{-}C}$ is further away from the plasma membrane than in EGFR. Based on this information, we believe that the lack of endocytosis for the heterodimer of EGFR and $ErbB2_{EGFR\text{-}C}$ is due to improper spatial coordination.

To further test this possibility, we shortened the EGFR intracellular domain by deleting 15 amino acids at the end of the kinase domain of EGFR to shift the C-terminal internalization codes closer to its transmembrane domain. This mutant EGFR (EGFRΔ943–957–FKBP) (Fig. 1E) contains an almost identical intracellular domain as wild-type EGFR, but its endocytic codes are shifted spatially closer to the transmembrane domain of EGFR. Thus, when this mutant heterodimerizes with wild-type EGFR, the heterodimer contains two sets of identical endocytic codes, but spatially, the coordination between the two sets of endocytic codes is compromised. We showed that, when expressed in 293T cells, this mutant EGFRΔ943–957–FKBP was internalized upon both EGF and AP20187 treatments as with wild-type EGFR (Fig. 8A–D). However, when co-expressed in 293T cells together with wild type EGFR–FRB, the internalization of heterodimer (EGFR–FRB)–(EGFRΔ943–957–FKBP) was significantly reduced upon stimulation by AP21967 (Fig. 8E–H). These results suggest that two sets of identical (compatible) endocytic codes are necessary, but not sufficient, to mediate EGFR endocytosis, and the spatial coordination of the two sets of identical (compatible) endocytic codes and perhaps other conditions are required to mediate the endocytosis of the receptor dimers.

DISCUSSION

Following our demonstration that EGFR endocytosis is driven by receptor dimerization rather than EGFR kinase activation (Wang et al., 2007; Wang et al., 2005), the goal of this study was to determine how dimerization regulates the endocytosis of EGFR. Based on our results, we propose that dimerization brings two sets of endocytic codes, one set of codes in each receptor, into close proximity, which then drives EGFR internalization. Here, we tested this hypothesis by using a receptor dimerization kit to specifically homodimerize or heterodimerize wild-type and mutant EGFRs and other receptor tyrosine kinases, including ErbB2, ErbB3 and PDGFRβ. The results are summarized in supplementary material Table S1. This system offers two advantages. First, this dimerization system can mimic the EGF-induced EGFR dimerization, and induce receptor kinase activation, phosphorylation and internalization. Second, by using this artificial dimerization system, we can specifically induce the formation of various receptor homodimers or heterodimers, permitting the test of our hypothesis by specifically studying the endocytosis of receptor homodimers and heterodimers.

It has been shown that EGFR internalization is largely dependent on the endocytic codes in the EGFR C-terminus domain (Chen et al., 1990; Canfield et al., 1991; Ohno et al.,

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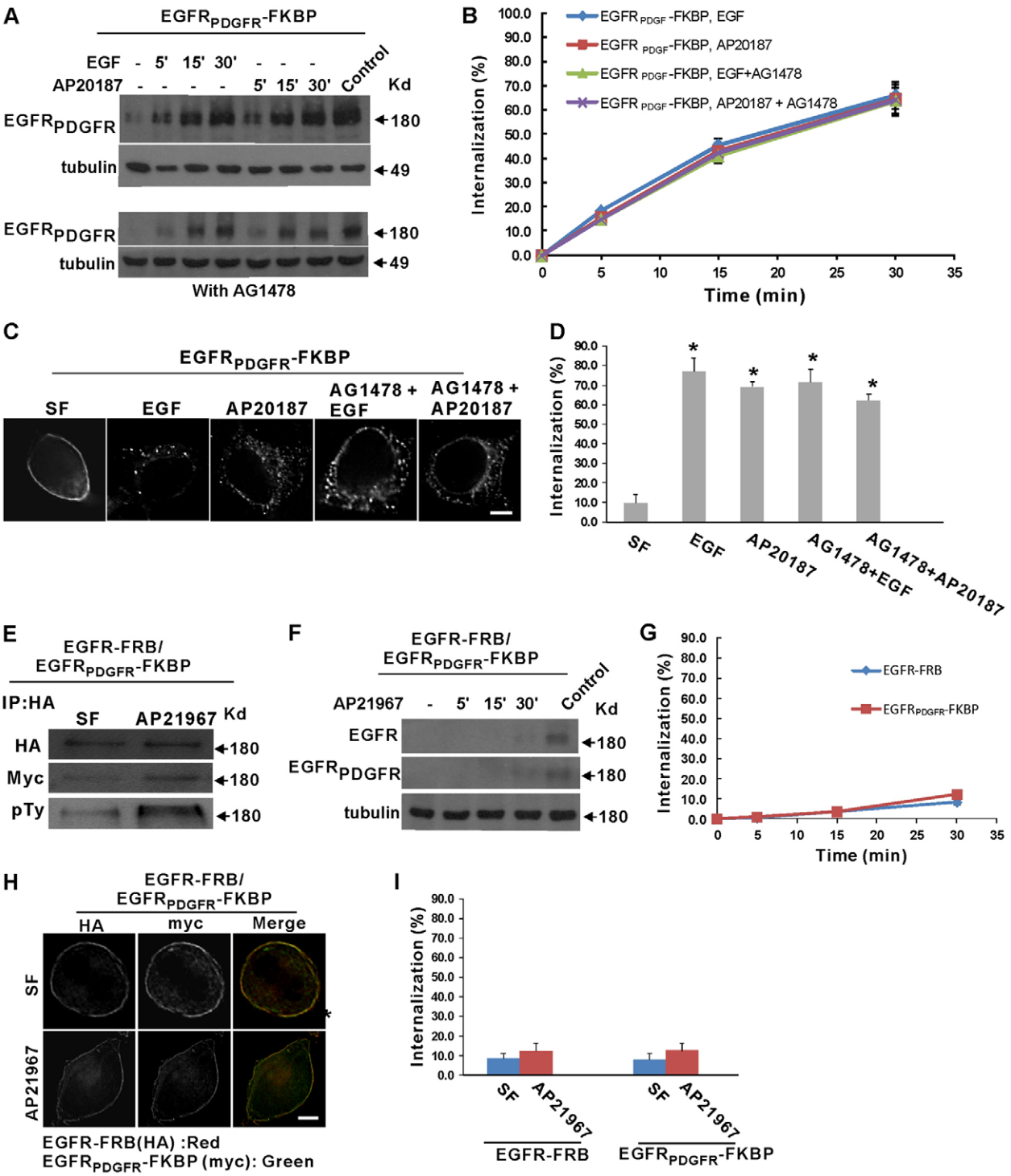

Fig. 5. Endocytosis of the $EGFR_{PDGFR}$–FKBP homodimer and its heterodimer with EGFR–FRB. (A–D) 293T cells were transiently transfected with $EGFR_{PDGFR}$–FKBP. The cells were then treated with EGF (50 ng/ml) or AP20187 (100 nM) with or without AG1478 for 30 min or as indicated. SF, serum free. (A) Endocytosis of $EGFR_{PDGFR}$–FKBP was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (B) Quantification of the data from A. Each value is the mean±s.e.m. of at least three experiments. (C) Endocytosis of $EGFR_{PDGFR}$-FKBP was examined by indirect immunofluorescence. Scale bar: 20 μm. (D) Quantification of data from C. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF. (E–I) 293T cells were co-transfected with EGFR–FRB and $EGFR_{PDGFR}$–FKBP. The cells were then treated with EGF (50 ng/ml) or AP20187 (100 nM) with or without AG1478 for 30 min or for the indicated time. (E) The association (dimerization) between EGFR–FRB and $EGFR_{PDGFR}$–FKBP and the phosphorylation of the receptors were examined by co-immunoprecipitation (IP). (F) Endocytosis of EGF–FRB and $EGFR_{PDGFR}$–FKBP heterodimers was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (G) Quantification of the data from F. Each value is the mean±s.e.m. of at least three experiments. (H) Endocytosis of EGF–FRB (red) and $EGFR_{PDGFR}$–FKBP (green) heterodimers was examined by double indirect immunofluorescence. Scale bar: 20 μm. (I) Quantification of data from H. Each value is the mean±s.e.m. of at least eight cells from three different experiments.

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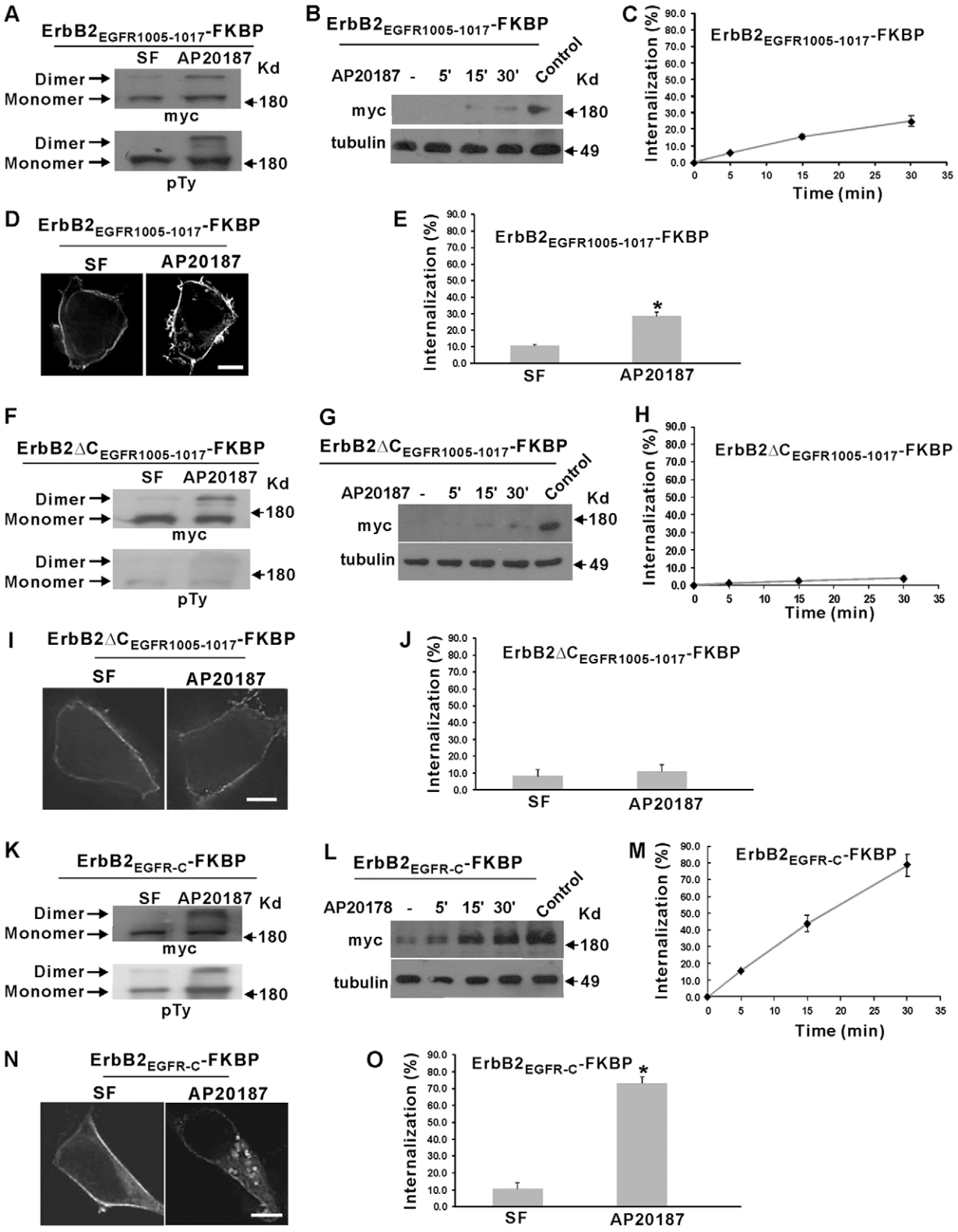

Fig. 6. See next page for legend.

1995; Mellman, 1996). Recently, we have shown that the EGFR C-terminal amino acid sequence 1005–1017, especially the dileucine motif $L^{1010}L^{1011}$, is an important endocytic code in mediating the endocytosis of EGFR dimers (Wang et al., 2007). It has also been reported that the impaired endocytosis of ErbB2 or ErbB3 homodimers is likely due to the lack of endocytic codes in their intracellular domains (Sorkin and Carpenter, 1993; Baulida et al., 1996; Muthuswamy et al., 1999; Wang et al., 1999). To test our above hypothesis stated, we examined whether two sets of endocytic codes are required for mediating the endocytosis of the

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Fig. 6. Endocytosis of the homodimers of various ErbB2 mutants fused with FKBP. (A–E) 293T cells were transiently transfected with $ErbB2_{EGFR1005–1017}$–FKBP. The cells were then treated with AP20187 (100 nM) for 30 min or as indicated. SF, serum free. (A) Dimerization and phosphorylation of $ErbB2_{EGFR1005–1017}$–FKBP were examined by crosslinking experiments followed by immunoblotting. (B) Endocytosis of $ErbB2_{EGFR1005–1017}$-FKBP was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (C) Quantification of the data from B. Each value is the mean±s.e.m. of at least three experiments. (D) Endocytosis of $ErbB2_{EGFR1005–1017}$–FKBP was examined by indirect immunofluorescence. Scale bar: 20 µm. (E) Quantification of data from D. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF. (F–J) 293T cells were transiently transfected with $ErbB2\Delta C_{EGFR1005–1017}$–FKBP. The cells were then treated with AP20187 (100 nM) for 30 min or as indicated. (F) Dimerization and phosphorylation of $ErbB2\Delta C_{EGFR1005–1017}$–FKBP were examined by crosslinking experiments followed by immunoblotting. (G) Endocytosis of $ErbB2\Delta C_{EGFR1005–1017}$–FKBP was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (H) Quantification of the data from G. Each value is the mean±s.e.m. of at least three experiments. (I) Endocytosis of $ErbB2\Delta C_{EGFR1005–1017}$–FKBP was examined by indirect immunofluorescence. Scale bar: 20 µm. (J) Quantification of data from D. Each value is the mean±s.e.m. of at least eight cells from three different experiments. (K–O) 293T cells were transiently transfected with $ErbB2_{EGFR-C}$–FKBP. The cells were treated with AP20187 (100 nM) for 30 min or as indicated. (K) Dimerization and the phosphorylation of $ErbB2_{EGFR-C}$–FKBP were examined by crosslinking experiments followed by immunoblotting. (L) Endocytosis of $ErbB2_{EGFR-C}$–FKBP was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (M) Quantification of the data from L. Each value is the mean±s.e.m. of at least three experiments. (N) Endocytosis of $ErbB2_{EGFR-C}$–FKBP was examined by indirect immunofluorescence. Scale bar: 20 µm. (O) Quantification of data from N. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF.

receptor dimers. We first showed that EGFR–ErbB2 heterodimers and EGFR–ErbB3 heterodimers are endocytosis deficient (Fig. 2). Given that these heterodimers only contain endocytic codes in one receptor, our data suggest that a pair of endocytic codes is required for the receptor endocytosis. We next examined the endocytosis of the (EGFR–FRB)–(EGFRΔ1005–1017–FKBP) heterodimer. Within this heterodimer, EGFR–FRB possesses complete endocytic codes and EGFRΔ1005–1017–FKBP lacks one endocytic code that is essential for its endocytosis. The fact that this heterodimer is impaired in endocytosis (Fig. 3) demonstrates that two sets of endocytic codes are required to mediate the endocytosis of receptor dimers.

We also found that to mediate the endocytosis of the receptor dimers, the two sets of endocytic codes must be compatible. This finding is supported by the following evidence. First, the heterodimer of EGFR and PDGFRβ is endocytosis deficient (Fig. 4), despite each receptor molecule containing endocytic codes that are capable of mediating the endocytosis of the receptor homodimers (Pahara et al., 2010; Wang et al., 2005; Wang et al., 2007). Second, we also constructed an EGFR and PDGFRβ swap mutant with a replacement of the EGFR endocytic amino acid code 1004–1017 with the corresponding PDGFRβ endocytic amino acid code 952–965 ($EGFR_{PDGFR}$–FKBP) (Wang et al., 2005; Wang et al., 2007; Pahara et al., 2010). We showed that the homodimer of this swap mutant $EGFR_{PDGFR}$–FKBP is endocytosed efficiently upon both EGF and AP20187 treatment (Fig. 5A–D). However, the heterodimer of this mutant with wild-type EGFR [(EGFR–FRB)–($EGFR_{PDGFR}$–FKBP)] is endocytosis deficient (Fig. 5E–I) despite the fact that the only difference between the two receptors within the heterodimer is the endocytic code (EGFR amino acids 1005–1017 compared with PDGFRβ amino acids 952–965).

Although we have shown previously that residues 1005–1017 and the $L^{1010}L^{1011}$ motif within this sequence are essential for EGFR endocytosis (Wang et al., 2007), we found here that they are not sufficient to mediate EGFR endocytosis, and other endocytic codes upstream of region 1005–1017 in C-terminus are also required (Fig. 6).

We have shown previously that the heterodimer of EGFR and a chimeric receptor composed of the ErbB2 extracellular domain and the EGFR intracellular domain is internalized following EGF-induced dimerization (Wang et al., 1999). Interestingly, we showed here that the EGFR–$ErbB2_{EGFR-C}$ heterodimer, which contains a pair of identical and intact endocytic codes, was still endocytosis deficient (Fig. 7F–J). As shown in Fig. 1, the EGFR C-terminus that contains the endocytic codes within $ErbB2_{EGFR-C}$ might be further away from the transmembrane domain of the receptor than within EGFR. Thus, one possible explanation for this impaired endocytosis is that the spatial coordination of the two sets of compatible endocytic codes is required. To further test this possibility, we constructed a mutant EGFR (EGFRΔ943–957–FKBP–HA) that contains almost the identical intracellular domain as the wild-type EGFR, but its C-terminus (which contains the endocytic codes) is shifted spatially closer to the transmembrane domain of EGFR. We showed that the internalization of the heterodimer (EGFR–FRB)–(EGFRΔ943–957–FKBP) was significantly reduced (Fig. 8). These results suggest that two sets of identical endocytic codes are necessary, but not sufficient, to mediate EGFR endocytosis. The spatial coordination of the two sets of compatible endocytic codes and perhaps other conditions are required. The spatial coordination between two receptor molecules has been implicated in the activation of receptor dimers (Bae and Schlessinger, 2010). The results of this study indicate that the spatial coordination between the endocytic codes of dimerized EGFR receptors is also a crucial factor in endocytosis.

Based on these data as summarized in supplementary material Table S1, we propose a ‘dimerization’ model to describe the regulation of EGF-induced EGFR endocytosis. In this model, the binding of EGF to EGFR induces the dimerization of EGFR, which results in the exposure of two sets of identical endocytic codes, one in each monomer. The downstream endocytic protein(s) required for EGFR endocytosis, might either be a dimer or have two identical binding sites and, thus, only interact with two identical and properly arranged endocytic codes simultaneously. Thus, in response to EGF, only EGFR homodimers undergo endocytosis, but EGFR–ErbB2 or EGFR–ErbB3 heterodimers cannot interact with the internalization-regulating protein(s) and fail to be internalized.

Endocytosis is an important way to downregulate cell surface receptors. Our model suggests that exploring the mechanisms that regulate endocytosis might yield key insights that would be useful for cancer therapy. It might be possible that a monoclonal antibody against the ErbB2 dimerization arm can inhibit its heterodimerization with other ErbB receptors, including EGFR and ErbB3, and therefore promote the formation of internalization-efficient EGFR homodimers and kinase-inactive ErbB3 homodimers, both of which would be helpful in reducing ErbB receptor signaling. Recently, Pertuzumab, a dimerization inhibitor for ErbB2, has been used in combination with other cancer therapies (Spiridon et al., 2002; Friedman et al., 2005). It

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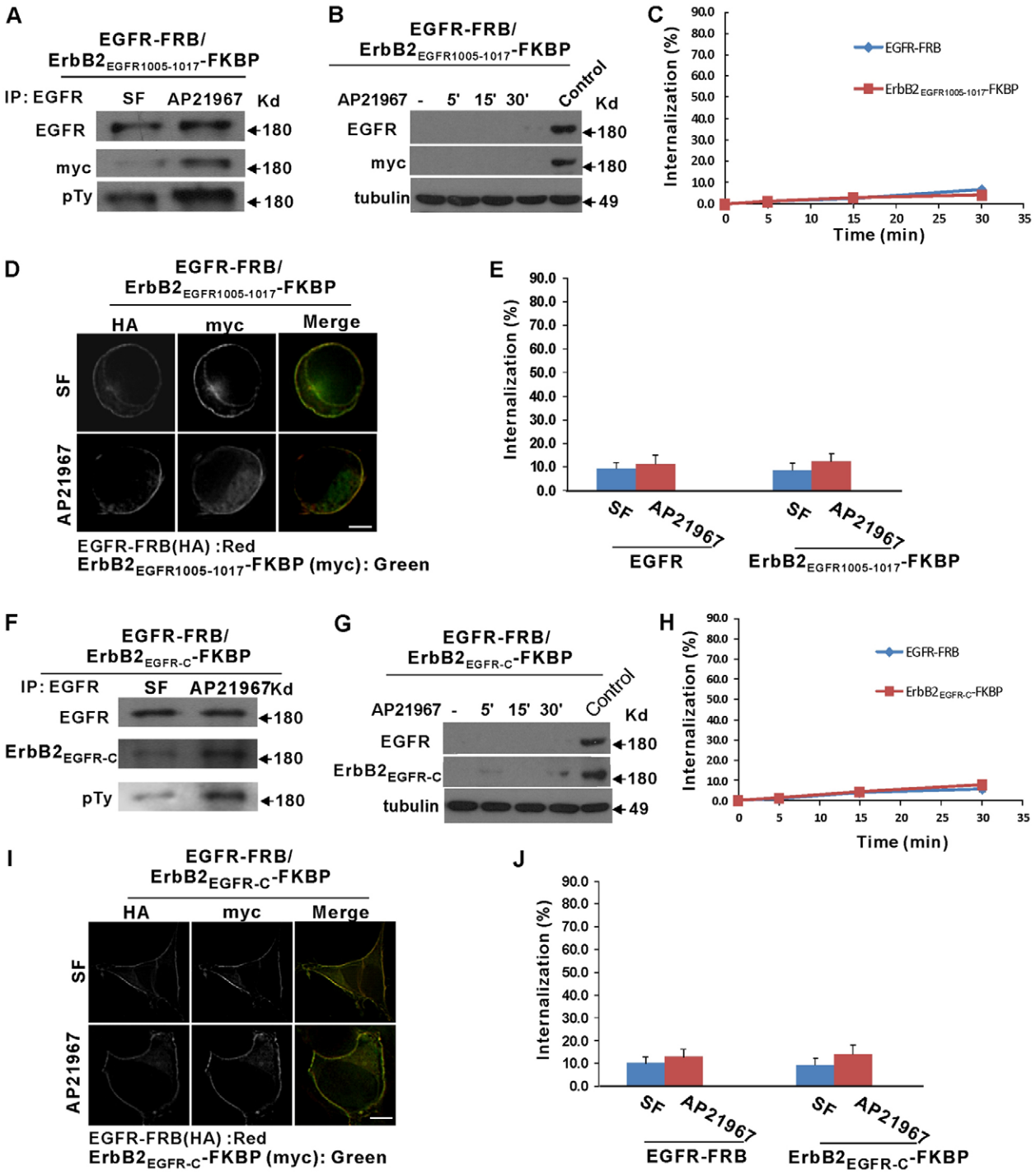

Fig. 7. Endocytosis of the heterodimers between EGFR–FRB and various ErbB2 mutants fused with FKBP. (A–E) 293T cells were transiently co-transfected with EGF–FRB and ErbB2$_{EGFR1005–1017}$–FKBP. The cells were then treated with AP21967 (500 nM) for 30 min or as indicated. SF, serum free. (A) Heterodimerization of EGF–FRB and ErbB2$_{EGFR1005–1017}$-FKBP. The association (dimerization) between EGFR–FRB and ErbB2$_{EGFR1005–1017}$–FKBP and the phosphorylation of EGFR–FRB were examined by co-immunoprecipitation (IP) and immunoblotting. (B) Endocytosis of EGF–FRB and ErbB2$_{EGFR1005–1017}$–FKBP heterodimers was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (C) Quantification of the data from B. Each value is the mean±s.e.m. of at least three experiments. (D) Endocytosis of EGF–FRB (red) and ErbB2$_{EGFR1005–1017}$–FKBP (green) heterodimers were examined by indirect immunofluorescence. Scale bar: 20 μm. (E) Quantification of data from D. Each value is the mean±s.e.m. of at least eight cells from three different experiments. (F–J) 293T cells were transiently co-transfected with EGF–FRB and ErbB2$_{EGFR-C}$–FKBP. The cells were then treated with AP21967 (500 nM) for 30 min or as indicated. (F) Heterodimerization of EGF–FRB and ErbB2$_{EGFR-C}$–FKBP. The association (dimerization) between EGFR–FRB and ErbB2$_{EGFR-C}$–FKBP and the phosphorylation of EGFR–FRB were examined by co-immunoprecipitation and immunoblotting. (G) Endocytosis of EGF–FRB and ErbB2$_{EGFR-C}$–FKBP heterodimers was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (H) Quantification of the data from G. Each value is the mean±s.e.m. of at least three experiments. (I) Endocytosis of EGF–FRB (red) and ErbB2$_{EGFR-C}$–FKBP (green) heterodimers were examined by double indirect immunofluorescence. Scale bar: 20 μm. (J) Quantification of data from I. Each value is the mean±s.e.m. of at least eight cells from three different experiments.

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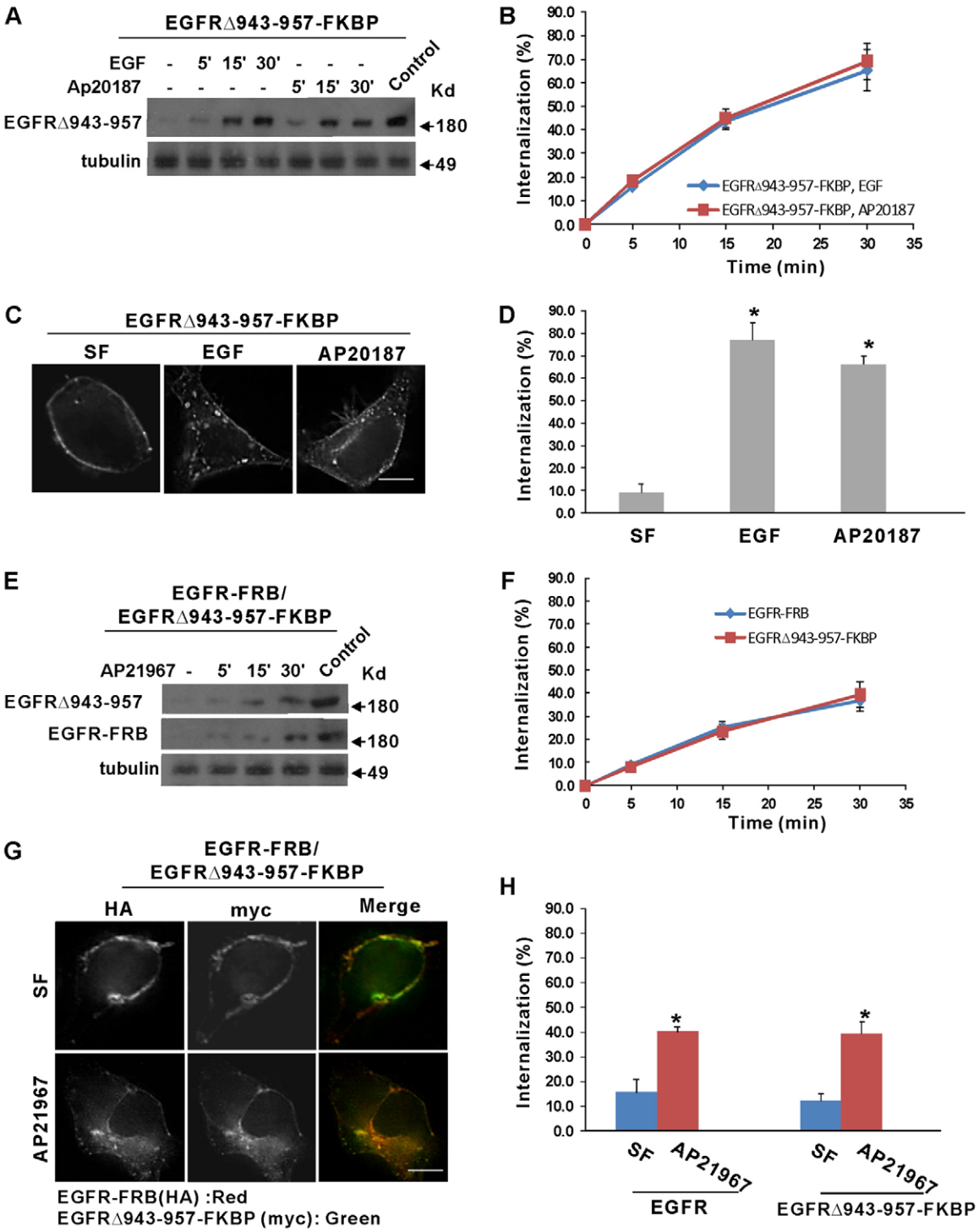

Fig. 8. Endocytosis of the EGFRΔ943–957–FKBP homodimer and its heterodimer with EGFR-FRB. (A–D) Endocytosis of the EGFRΔ943–957–FKBP homodimer. 293T cells were transiently transfected with EGFRΔ943–957-FKBP. The cells were treated with EGF (50 ng/ml) or AP20187 (100 nM) for 30 min or as indicated. SF, serum free. (A) The endocytosis of EGFRΔ943–957–FKBP was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (B) Quantification of the data from A. Each value is the mean±s.e.m. the of at least three experiments. (C) Endocytosis of EGFRΔ943–957–FKBP was examined by indirect immunofluorescence. Scale bar: 20 μm. (D) Quantification of data from C. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF. (E–H) Endocytosis of the heterodimers between EGFRΔ943–957–FKBP and EGFR–FRB. 293T cells were co-transfected with EGF–FRB and EGFRΔ943–957–FKBP. The cells were treated with AP21967 (500 nM) for 30 min or as indicated. (E) Endocytosis of EGF–FRB and EGFRΔ943–957–FKBP heterodimers was examined by following the cell surface biotinylation signal. Control is as described in the legend for Fig. 2B. (F) Quantification of the data from E. Each value is the mean±s.e.m. of at least three experiments. (G) Endocytosis of EGF–FRB (red) and EGFRΔ943–957–FKBP (green) heterodimers by double indirect immunofluorescence. Scale bar: 20 μm. (H) Quantification of data from G. Each value is the mean±s.e.m. of at least eight cells from three different experiments. *$P<0.01$ when compared with SF.

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has also been reported that peptide vaccines of the ErbB2 dimerization loop are effective in inhibiting mammary tumour growth *in vivo* (Allen et al., 2007).

Many receptors, including PDFGR, nerve growth factor receptor and hepatocyte growth factor receptor, are dimerized and internalized after ligand binding (Oved and Yarden, 2002; Schlessinger, 2002). Thus, it is possible that our findings for ErbB receptors are also applicable to these receptor tyrosine kinases.

MATERIALS AND METHODS

Antibodies and chemicals

The plasmids used in this research include pcDNA3.1/Myc-His(-)A (Invitrogen, Burlington, ON), pC4-Fv1E and pC4-R_HE (Ariad Pharmaceuticals, Cambridge, MA), pEYFP-N1 (Mountain View, CA), and pRSET-B-mCherry (a gift from Roger Tsien, University of California San Diego, CA). Mouse monoclonal antibodies against ErbB2 (9G6), Myc (9E10), PDGFRβ and phosphorylated tyrosine (pY99), and rabbit polyclonal antibodies against EGFR(1005), ErbB2 (C18), ErbB3 (C17), and HA (Y11) were obtained from Santa Cruz Biotechnology, CA. Mouse monoclonal antibodies against EGFR (Ab-1) were obtained from Oncogene Research Products (Boston, MA). Unless otherwise specified, all of the chemicals and reagents were obtained from Sigma (St. Louis, MO).

Cell culture, treatment and transfection

293T cells (human embryonic kidney cells, ATCC CRL-11268) were grown at 37℃ in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin, and streptomycin (100 U/ml) and were maintained in a 5% CO_2 atmosphere.

All of the receptors and mutants were expressed in 293T cells by transient transfection with calcium phosphate. To specifically induce receptor homodimerization or heterodimerization, the transfected cells were treated with 100 nM AP20187, 500 nM AP21967, EGF (50 ng/ml) or HRG (50 ng/ml) for 30 min or as indicated.

Regulated receptor homo- and hetero-dimerization

To develop a controllable and regulated receptor homo- and hetero-dimerization system that allowed us to specifically study the internalization of receptor homodimers or heterodimers, the 'ARGENT^{TM} Regulated Homodimerization kit' and 'ARGENT^{TM} Regulated Heterodimerization kit' from ARIAD Pharmaceuticals, Inc. (Cambridge, MA) were used. In this system, the chemicals specifically homodimerize proteins fused with FKBP or heterodimerize two proteins fused with FKBP and FRB respectively (Keenan et al., 1998; Stockwell and Schreiber, 1998; Muthuswamy et al., 1999; Wilde et al., 1999; Neff and Blau, 2001; Welm et al., 2002; Clackson, 2006; Zhan et al., 2006).

Regulated receptor homo- and hetero-dimerization assays were carried out according to the instructions of the manufacturer. Briefly, we constructed a series of receptor chimeras by fusing FKBP or FRB to the C-terminus of different receptors like EGFR, ErbB2, ErbB3, and some mutant receptors using molecular cloning techniques. Following transfection of FKBP fusion receptors or FKBP and FRB fusion receptors, 293T cells were serum-starved for 24 h and then treated with homodimerizer AP20187 (100 nM) or heterodimerizer AP21967 (500 nM) for 30 min at 37℃.

Plasmid construction

All the constructs used in this research are shown in Fig. 1. To construct mCherry-tagged wild-type EGFR, the wild-type EGFR gene was amplified from pEYFP-N1/EGFR (previously constructed in our laboratory) by PCR and inserted into the pRSET-B-mCherry vector (gift from Roger Tsien) using the *KpnI/AgeI* sites. To construct FKBP- or FRB-fused EGFR, ErbB2 and ErbB3, full-length EGFR, ErbB2 and ErbB3 were amplified from pEYFP-N1/EGFR, pcDNA3.1/Myc-His(-)A/ErbB2, and pcDNA3.1/Myc-His(-)A/ErbB3, respectively, then subcloned into the pC4-Fv1E vector (Ariad Pharmaceuticals) to make FKBP fusion proteins, or the pC4-R_HE vector (Ariad Pharmaceuticals) to make FRB fusion proteins. All the FKBP fusion proteins were epitope-tagged with HA and the FRB fusion protein was epitope-tagged with Myc. To construct different deletion mutants of EGFR, such as FKBP-fused EGFRΔ943–957, upstream and downstream primers were designed to be complementary to the nucleotide sequences adjacent to and ahead of the deletion fragments, respectively. Then, the whole plasmids were amplified directly from pEYFP-N1/EGFR or pC4-Fv1E/EGFR plasmids previously made using Pfu polymerase, and finally, self-ligated.

To construct the ErbB2 mutant, $ErbB2_{EGFR1005–1017}$–FKBP, with the insertion of the EGFR 1005–1017 amino acid fragment, upstream and downstream primers were designed to be complementary to the nucleotide sequences adjacent to and ahead of the insertion site, and contained the 1005–1017 sequence at the 5′ end. Then, the whole mutant plasmids were amplified directly from pC4-Fv1E/ErbB2 plasmids using Pfu polymerase and finally, self-ligated. To construct the FKBP-fused ErbB2 chimera, $ErbB2_{EGFR-C}$–FKBP, the previously made pcDNA3.1/Myc-His(-)A/$ErbB2_{EGFRc\text{-}terminus}$ was used as template.

Cross-linking assay

293T cells were cultured in 60-mm dishes to subconfluency. Following transfection with constructs encoding wild-type or mutated receptors, the cells were serum-starved for 24 h. After treatment with specific ligands for 15 min or homodimerizer AP20187 for 30 min at 37℃, the cells were collected in 0.2–0.5 ml PBS. Crosslinking reagents bis(sulfosuccinimidyl)suberate (BS3) or disuccinimidyl suberate (DSS) (Calbiochem) were then added to a final concentration of 1.0–2.5 mM, and the reaction was incubated on ice for 2 h. The quench solution (1 M Tris-HCl pH 7.5, 1:100 dilution) was then added to a final concentration of 10 mM and incubated for 15 min on ice. The cells were then lysed with 1% NP-40 for 1 h on ice, and receptor dimerization was analyzed by SDS-PAGE and immunoblotting.

Cell lysates and immunoblotting

To obtain total cell lysates, the cells were collected, lysed and homogenized in ice-cold Mammalian Protein Extraction Reagent (Pierce, Rockford, Illinois) containing a phosphatase and protease inhibitor cocktail [4 mM NaF, 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 µg/ml aprotinin, 1 µM pepstatin A]. The lysates were then centrifuged at 21,000 ***g*** at 4℃ for 30 min. The supernatant was collected, the protein concentration was quantified and the sample was boiled in SDS loading buffer for 5 min. Immunoblotting was performed as previously described (Wang et al., 2002).

Immunoprecipitation

Immunoprecipitation experiments were carried out as described previously (Wang et al., 1999). Briefly, cells were lysed with immunoprecipitation buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 100 mM NaF, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 µg/ml aprotinin, and 1 µM pepstatin A] and the lysate was centrifuged to remove debris. The supernatants, containing 1 mg of total protein, were incubated with 1 µg of specific antibody. Then, goat anti-mouse-IgG conjugated to agarose, or protein A conjugated to agarose was added to each fraction. For the controls, pre-immune mouse or rabbit IgG were used to replace the primary antibodies. The samples were boiled for 5 min and analyzed by SDS-PAGE followed by western blotting.

Cell surface biotinylation assay to examine receptor internalization

To examine the internalization of the receptors, cell surface biotinylation assays, as we previously described, were used in this study (Pahara et al., 2010). To achieve the selective labeling and removal of only the cell surface proteins, the cleavable, water-soluble and membrane-impermeable biotin analog sulfo-NHS-SS-biotin was used. Then, free sulfo-NHS-SS-biotin was quenched by NH_4Cl. Briefly, cells were incubated with 0.5 mg/ml sulfo-NHS-SS-biotin in PBS/CM (10 mM

Na_2HPO_4, 2 mM KH_2PO_4 pH 7.2) on ice for 30 min with rocking. Then, the cells were incubated three times with 50 mM NH_4Cl in PBS/CM on ice for 5 min to quench the free biotin. The cells were then stimulated with EGF for 0, 5, 15 and 30 min. Following incubation with 100 mM MESNA at 4℃ for 10 min to remove biotin from the sulfo-NHS-SS-biotin-labeled proteins on the cell surface, cells were treated with 5 mg/ml iodoacetamide in PBS/CM on ice for 5 min to quench free SH groups. Endocytosis was detected by the accumulation of the sulfo-NHS-SS-biotin-labeled cargo proteins within the cells, which were protected from reduction by MESNA. The cells that were biotinylated but not treated with MESNA were used as a control to show the total biotinylated receptors. Cells were then lysed and the biotinylated proteins were precipitated using NeutrAvidin beads. The amount of internalized and total biotinylated receptors were determined by immunoblotting with antibody to the receptors. For the quantification, the internalization of the recepotors was determined by the ratio between internalized biotinylated receptor and total biotinylated receptor, and expressed as percentages. Each value is the mean±s.e.m. of at least three experiments. Significance was calculated by using Student's *t*-test.

Fluorescence microscopy and the quantification of receptor endocytosis

Indirect immunofluorescence microscopy was carried out as described previously (Wang et al., 1999). For the quantification of receptor internalization, ~14 images of a cell along the *z*-axis were acquired and the images were deconvolved by DeltaVision softWoRx software. The boundary of a cell in each *z*-section was determined by using differential interference contrast (DIC) microscopy. In each image (a *z*-section of the cell), one polygon (larger polygon) was drawn along the outer edge of the cell membrane to represent the entire cell area in that section and the other polygon (smaller polygon) drawn along the inner edge of the cell membrane representing the cell interior. The fluorescence intensity of the receptors in these two polygons areas was measured. The value of the large polygon (V_L) represents the total fluorescence intensity of the receptors on both the cell surface and interior of the cell in the section. The value of the small polygon (V_S) represents the fluorescence intensity of the internalized receptors in the section. The difference in the fluorescence intensity between the large polygon and small polygon areas ($V_L - V_S$) represents the fraction of the studied receptors remaining on the membrane in the section. Thus, the percentage of receptor internalization in the cell is calculated as:

$$\text{Internalized receptor } (\%) = \frac{\text{Sum of } V_S \text{ in all the } Z\text{-sections}}{\text{Sum of } V_L \text{ in all the } Z\text{-sections}} \times 100\%$$

For each value, at least eight cells from three experiments were analyzed and results are mean±s.e.m. Significance was calculated by using Student's *t*-test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Q.W. designed and performed most experiments and wrote the first draft of the paper. X.C. performed some experiments. Z.W. wrote the final version of the paper.

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Supplementary material

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