Interaction of EGF receptor and Grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy

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The interaction of activated epidermal growth factor receptor (EGFR) with the Src homology 2 (SH2) domain of the growth-factor-receptor binding protein Grb2 initiates signaling through Ras and mitogen-activated protein kinase (MAP kinase) [1,2]. Activation of EGFRs by ligand also triggers rapid endocytosis of EGF–receptor complexes. To analyze the spatiotemporal regulation of EGFR–Grb2 interactions in living cells, we have combined imaging microscopy with a modified method of measuring fluorescence resonance energy transfer (FRET) on a pixel-by-pixel basis using EGFR fused to cyan fluorescent protein (CFP) and Grb2 fused to yellow fluorescent protein (YFP). Efficient energy transfer between CFP and YFP should only occur if CFP and YFP are less than 50 Å apart, which requires direct interaction of the EGFR and Grb2 fused to these fluorescent moieties [3]. Stimulation by EGF resulted in the recruitment of Grb2–YFP to cellular compartments that contained EGFR–CFP and a large increase in FRET signal amplitude. In particular, FRET measurements indicated that activated EGFR–CFP interacted with Grb2–YFP in membrane ruffles and endosomes. These results demonstrate that signaling via EGFRs can occur in the endosomal compartment. The work also highlights the potential of FRET microscopy in the study of subcellular compartmentalization of protein–protein interactions in living cells.

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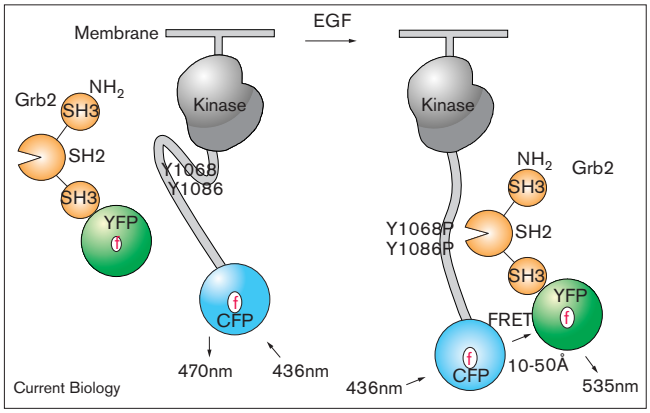
Figure 1



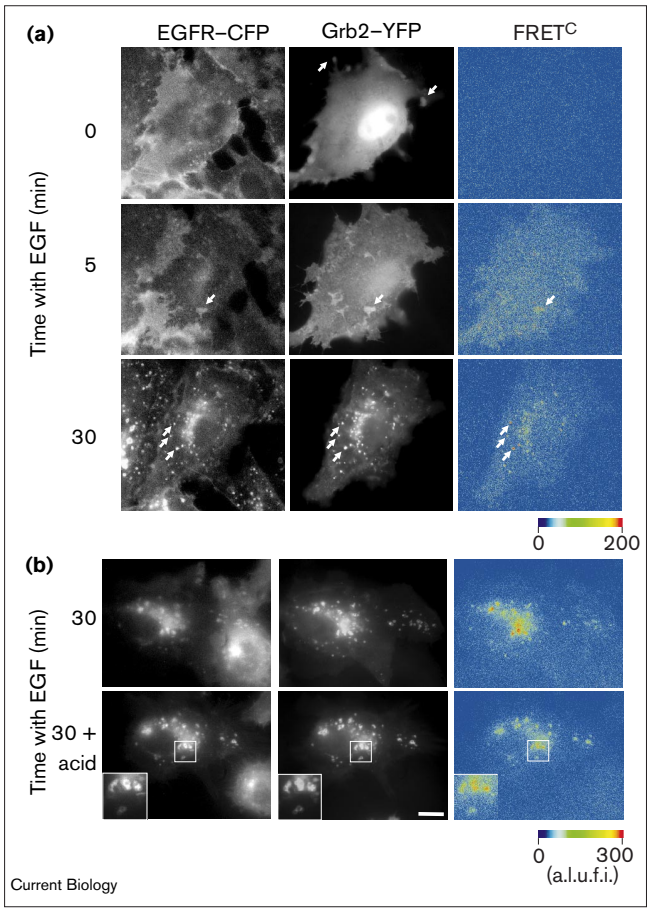
Hypothetical model of EGFR–Grb2 interaction measured by FRET. In our FRET registration system, the excitation of CFP at 436 nm will result in emission at 470 nm if no YFP is in close proximity. If the distance between CFP and YFP is less than 50 Å, however, due to formation of the EGFR–CFP/Grb2–YFP complex, then the energy will be transferred from excited CFP to YFP, resulting in the sensitized emission at 535 nm. The figure shows a possible arrangement of the intracellular domain of EGFR–CFP associated with Grb2–YFP via phosphorylated Tyr1068 and Tyr1086 [9]. The SH2 and SH3 domains of Grb2, EGFR kinase, YFP and CFP are drawn according to their approximate sizes deduced from the three-dimensional structures. f (red), fluorophores of YFP and CFP.

Results and discussion

We have previously generated a functional chimera of EGFR and green fluorescent protein (GFP) [4]. EGFR–GFP can activate MAP kinase, suggesting that the initial step of this signaling pathway, the receptor–Grb2 interaction, occurs normally. These experiments opened the way for use of chimeric EGFRs and FRET microscopy to analyze receptor interactions with Grb2 in living cells. EGFR–CFP was stably expressed in porcine aortic endothelial (PAE) cells that lack endogenous EGFRs [4]. Chimeric Grb2–YFP was generated by fusion of YFP to the carboxyl terminus of Grb2 (Figure 1) and transiently expressed in PAE/EGFR–CFP cells. To analyze the effect of EGF on subcellular distribution and interactions of EGFR–CFP and Grb2–YFP, digital images were acquired through CFP, YFP and FRET channels from a single cell at successively longer elapsed times following stimulation with EGF (Figure 2). Corrected FRET ($FRET^C$) was calculated for the entire image on a pixel-by-pixel basis using a three-filter 'micro FRET' method [5] and is presented as a quantitative pseudocolor image.

Calculation of $FRET^C$ includes corrections for the background fluorescence and for the fact that raw FRET images consist of both FRET and non-FRET components resulting from the cross-over of donor and acceptor fluorescence through the FRET filters. Cross-over can be calculated as a constant proportion of the CFP and YFP

Figure 2



Interactions of EGFR–CFP and Grb2–YFP in membrane ruffles and endosomes measured by FRET. Grb2–YFP was transiently expressed in PAE/EGFR–CFP cells. Cells were incubated with 200 ng/ml EGF for 5–30 min at 37°C. **(a)** Images were acquired at 20°C with CFP (left column), YFP (central column) and FRET (data not shown) filter sets before (time 0) and after a 37°C incubation of cells with EGF. **(b)** Cells incubated with EGF for 30 min were treated with mild acidic buffer (0.2 M sodium acetate, 0.5 M NaCl pH 4.5) for 2 min at 4°C to dephosphorylate surface EGFR–CFP followed by image capture from the same cell at room temperature (30 + acid). YFP and CFP images have been background-subtracted. EGFR–CFP and Grb2–YFP localization in lamellipodia, ruffles and endosomes are indicated by arrows. Inserts show high-resolution images of endosomes containing EGFR–CFP and Grb2–YFP. Binning 2 × 2 (a) or 'no binning' mode (b) (increased resolution compared to (a)) were used. $FRET^C$ images are displayed using quantitative pseudocolor (right column). a.l.u.f.i, arbitrary linear units of fluorescence intensity. The scale bar represents 10 μm.

fluorescence through the CFP and YFP channels, respectively. The extent of cross-over is characteristic of the particular optical system and does not depend on whether both YFP and CFP are present; it can be determined using cells that express either EGFR–CFP or Grb2–YFP. Our measurements revealed that 55.5 ± 0.5% of CFP and 1.25 ± 0.05% of YFP fluorescence can bleed through the FRET channel. Therefore, to calculate the cross-talk in our experiments, CFP and YFP images were multiplied by, respectively, 0.560 and 0.015 (these numbers allow 'conservative' calculations of $FRET^C$). Finally, the $FRET^C$ image was obtained by subtracting CFP and YFP cross-over from raw FRET images.

Under steady-state conditions (no EGF) EGFR–CFP was diffusely distributed throughout the cell, and a pool of newly synthesized and constitutively internalized receptors were located in the Golgi area and vesicular structures (Figure 2a, time 0). Grb2–YFP was also distributed throughout the cell with pronounced nuclear accumulation ([6], and Figure 2a). In addition, Grb2–YFP was seen concentrated in lamellipodia. Immunofluorescence staining revealed a similar pattern of localization of endogenous Grb2 (Figure 3). Apparently, localization of Grb2–YFP to nuclei was due to weak and readily reversible interactions because Grb2–YFP moved out of the nuclei immediately upon activation of EGFRs. $FRET^C$ was minimal in all regions of cells that were not stimulated with EGF (Figure 2a). In particular, FRET signals were not observed in the Golgi area enriched with EGFR–CFP nor in cell nuclei containing large amounts of Grb2–YFP. This validates the accuracy of our method of correction for non-FRET component of the FRET images.

Incubation of cells with EGF at 37°C led to rapid redistribution of both tagged proteins. Within 1–5 minutes, EGFR–CFP and Grb2–YFP fluorescence became co-localized in plasma membrane ruffles and in small vesicular structures, presumably early endosomes (Figure 2a). The localization of microinjected Grb2 in the membrane ruffles was detected by immunofluorescence staining in fixed cells [7]. In living cells, the appearance of Grb2–YFP in ruffles was much more dramatic after cell stimulation with EGF.

After 10–30 minutes of EGF stimulation, both EGFR–CFP and Grb2–YFP became concentrated in large perinuclear endosome-like vesicles (Figure 2a). These endosomes also contained transferrin receptors and/or EEA.1 protein, markers of early/intermediate endosomal compartments (data not shown). At all times during incubation with EGF, a high degree of co-localization of Grb2–YFP with the EGFR–CFP was observed, suggesting that Grb2 is recruited to the sites of activated EGFRs. Grb2–YFP mutants with an impaired binding capacity in the SH2 domain did not leave nuclei and move to ruffles, although a small amount of mutant Grb2–YFP was seen in endosomes (data not shown). Immunofluorescence staining revealed accumulation of endogenous Grb2 on endosomes containing activated EGFR–CFP (Figure 3). Similarly, Grb–YFP was recruited to endosomes in EGF-stimulated PAE cells expressing wild-type EGFR (Figure 3). These data suggest that the YFP and CFP moieties do not affect trafficking of EGFR and Grb2.

Table 1

Normalized FRET in individual compartments and regions of cells.

Time with EGF (min)	$FRET^{C}$*	$FRETN^{C/CFP}$	$FRETN^{C/YFP}$ ($\times 10^{-3}$)	$FRETN^{C/CFP \times YFP}$ ($\times 10^{-5}$)
0†	12 ± 10	0.14 ± 0.06	2.0 ± 0.6	1.9 ± 0.8
5‡	120 ± 15	0.50 ± 0.06	18.1 ± 0.2	8.8 ± 0.7
20–60§	218 ± 27	0.83 ± 0.08	13.2 ± 2.6	5.4 ± 1.4

*$FRET^{C}$ values are presented in arbitrary linear units of fluorescence intensity. Normalized FRET values were calculated by dividing $FRET^{C}$ for each selection by the mean intensity of YFP, CFP or both YFP and CFP in the subregion to yield $FRETN^{C/YFP}$, $FRETN^{C/CFP}$ and $FRETN^{C/(YFP \times CFP)}$, respectively. †Subregions of diffuse fluorescence, Golgi area and Grb2–YFP accumulations at the cell edges were selected. ‡Membrane ruffles and subregions of the diffuse fluorescence were selected. §Endosomes were selected. Values are presented ± standard deviation.

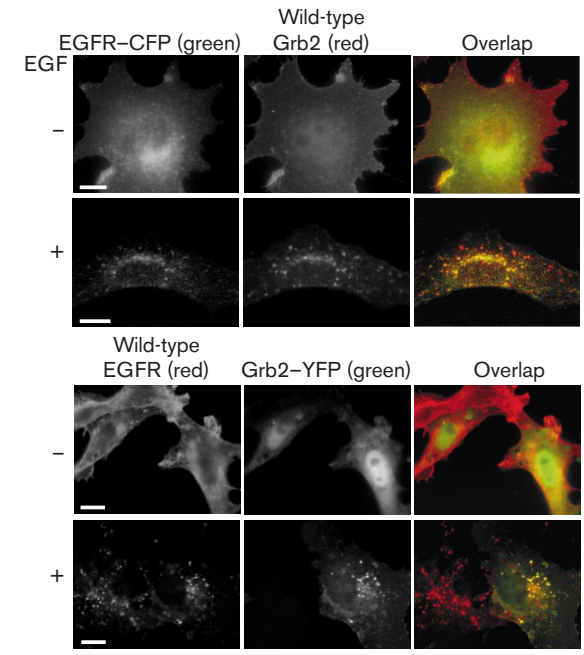
$FRET^{C}$ was detected immediately after EGF stimulation. Initially, the highest $FRET^{C}$ values were obtained from areas of co-localization of EGFR–CFP and Grb2–YFP in membrane ruffles (Figure 2a, 5 minutes). At later times, the strongest FRET signals were observed in large perinuclear endosomes (Figure 2, 30 minutes). To test whether interacting EGFR–CFP and Grb2–YFP were indeed located in endosomes, EGF was removed from the cell surface with mild acid wash to deactivate and dephosphorylate surface EGFR–CFP without affecting phosphorylation of internalized EGFR–CFP [8]. Such treatment should selectively dissociate surface EGFR/Grb2 complexes. As shown in Figure 2b, the efficient FRET was observed in the same vesicular structures after acid wash, indicating that the EGFR–CFP/Grb2–YFP complexes were acid-resistant and, therefore, located intracellularly.

To compare FRET efficiencies in different cellular compartments, $FRET^{C}$ was normalized to the amount of donor and/or acceptor; normalized $FRET^{C}$ is denoted $FRETN^{C}$. To this end, mean intensities of YFP, CFP and FRET were measured for a large number of selected cellular subregions with background subtracted. $FRET^{C}$ values were calculated by correcting raw FRET intensities for the cross-over of YFP and CFP fluorescence. $FRET^{C}$ values were then normalized to the mean fluorescence intensity of CFP and/or YFP in the selected subregion to obtain $FRETN^{C}$. In control cells (no EGF), very weak $FRETN^{C}$ signals were detected associated with the peripheral areas (Table 1) and no FRET was detected from cell nuclei (data not shown). In contrast, significantly higher values of $FRETN^{C}$ were obtained for membrane ruffles and endosomes in EGF-stimulated cells. It is still debatable which method of $FRET^{C}$ normalization is most appropriate for the quantitative analysis of the dynamic interaction of two proteins present in an unknown stoichiometry. In general, it appears to be preferable to normalize the $FRET^{C}$ signal to the amount of donor present [5]. In our experiments, however, it is logical to relate $FRET^{C}$ to the amount of an acceptor, YFP, because Grb2–YFP may be a limiting component that determines the amount of the EGFR–CFP/Grb2–YFP complexes. But given that Grb2 can bind to the EGFR through Shc [9] in addition to direct binding, the number of Grb2–YFP molecules per selected subregion could be greater than the amount of EGFR–CFPs in the subregion. In fact, Shc could be readily detected by immunofluorescence staining in endosomes containing EGFR–CFP and Grb2–YFP (data not shown).

The data obtained using 'channel math' (Figure 2) and regional analysis methods (Table 1) strongly indicate that the CFP moiety attached to the EGFR and the YFP moiety attached to Grb2 remain in close proximity to each other as the fusion proteins traverse distinct cellular compartments. Thus, FRET experiments show for the first time interaction between EGFR and Grb2 in living cells, both at the cell surface and after receptor internalization in endosomes. The role of endocytosis in growth factor signaling remains controversial [10]. The presence of active EGFRs and their potential to trigger mitogenic signals has been clearly shown. In particular, Grb2, Shc and EGFR co-immunoprecipitation in rat liver endosomal fractions has been reported [11]. Endocytosed EGFRs have been implicated in the activation of the MAP kinase pathway in HeLa cells [12]. In other cells, however, EGFR endocytosis is not required for MAP kinase activation [13,14]. Our experiments argue that EGFRs are capable of signaling for a long time after EGF stimulation. PAE/EGFR–CFP cells have about 10^6 EGF-binding sites per cell. This expression level is comparable to that in many squamous and epidermoid carcinoma cells. Thus, the persistence of EGFR–Grb2 complexes in endosomes in our model system may mimic the situation in these cancerous cells.

Fluorescence lifetime imaging was recently used to demonstrate phosphorylated internalized EGFR–GFP in chemically fixed cells [15]. This technique could not be used for pixel-by-pixel analysis of CFP–YFP interactions in living cells because of movement of endosomes and significant photobleaching of CFP (10–15%) during photobleaching of YFP. Our approach allowed time-dependent measurement of FRET signals between two proteins tagged with YFP and CFP on a pixel-by-pixel basis and in living cells. Unlike the conventional two-filter method of measuring the ratio of fluorescence intensities through the FRET and CFP filter sets [16,17], the three-filter method does not require comparison of two experimental conditions for FRET detection and takes into account YFP fluorescence cross-over [5]. This method can potentially be used to analyze EGFR interactions with other signaling and trafficking proteins. Obvious obstacles are the possibility of ineffective fluorochrome orientation and of YFP and CFP being too far apart in other protein–protein pairs. For

Figure 3



Localization of endogenous Grb2 and wild-type EGFR in PAE cells. PAE/EGFR–CFP were incubated with EGF for 30 min at 37°C, fixed with paraformaldehyde and stained with monoclonal antibodies to Grb2, whereas PAE cells stably expressing wild-type EGFR (PAE/wtEGFR) and transiently expressing Grb2–YFP were stimulated with EGF for 30 min, fixed with paraformaldehyde, and stained with monoclonal antibody to EGFR. Both monoclonal antibodies were detected with secondary CY3-conjugated antibodies. A *z*-stack of YFP or CFP (green) and CY3 (red) images were acquired from EGF-stimulated and non-stimulated cells and deconvoluted as described [18]. Individual optical sections are presented. Superimposed images are shown in the right column. Scale bars represent 10 μm.

example, no FRET was detected in EGF-stimulated cells coexpressing EGFR–CFP with EGFR–YFP or ErbB2–YFP, which indicates unfavorable orientation of YFP and CFP in the EGFR homo- and heterodimers (data not shown). Nevertheless, the simplicity of measurement described here allows rapid testing of the feasibility of using FRET microscopy to analyze subcellular localization of any protein–protein interaction in living cells.

Materials and methods

PAE/EGFR–CFP cells transiently expressing Grb2–YFP were plated onto ΔTC3 microscopy dishes (Bioptech). Dishes were mounted on the microscope stage using a temperature-controlled stage adaptor. To measure FRET, three images were acquired in the same order in all experiments through YFP, CFP and FRET filters using the same dual-band beamsplitter. Full details of techniques are available in Supplementary material.

Supplementary material

Supplementary material including generation and expression of EGFR–CFP and Grb2–YFP and Imaging workstation, filter set parameters, image acquisition procedure and calculations of corrected and normalized FRET is available at http://current-biology.com/supmat/supmatin.htm.

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