Role of Grb2 in EGF-stimulated EGFR internalization

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Summary

Grb2 is an adaptor molecule that couples membrane receptors such as the epidermal growth factor receptor (EGFR) to intracellular signaling pathways. To gain insight into the trafficking pathways followed by these molecules after activation by EGF, we visualized Grb2 and EGFR fused to GFP spectral variants in single live cells. In nonstimulated cells, Grb2-YFP was primarily localized diffusely in the cytoplasm, whereas EGFR-CFP was found on the plasma membrane and in endocytic structures localized in the perinuclear area. Within 1 minute of EGF stimulation, Grb2 redistributed to the plasma membrane where it bound EGFR-CFP in an SH2 dependent manner. The plasma membrane then began to dynamically ruffle, and Grb2-YFP and EGFR-CFP were found to internalize together in large macropinocytic structures. These structures were morphologically distinct from conventional, clathrin-derived endosomes and did not label with transferrin, AP-2 or clathrin heavy chain. Evidence that these structures did not require clathrin for internalization came from experiments showing that expression of the C-terminus of AP-180, which inhibited transferrin uptake, had no effect on EGF-induced internalization of EGFR. YFP-tagged Grb2 containing an inhibitory mutation in either N- or C-SH3 domain redistributed to the plasma membrane upon EGF stimulation, but the macropinocytic structures containing Grb2-YFP and EGFR-CFP did not translocate inward and appeared to remain tethered to the plasma membrane. This suggested that the Grb2 SH3 domain was responsible for coupling the membranes involved in internalization of EGFR with downstream effectors involved in internalization of these membranes. Transferrin uptake was unaffected in the presence of all of the SH3 domain mutants, consistent with the EGF-stimulated EGFR internalization pathway being clathrin-independent. These results demonstrate a role for Grb2 in events associated with a macropinocytic internalization pathway for EGFR in activated cells.

Movies available on-line

Key words: Grb2, EGFR, SH3 domain, Endocytosis, GFP

Introduction

Adaptor molecules are modular protein structures containing protein and lipid binding domains, and stretches of sequence to which some of these domains bind (Pawson, 1995). Adaptors are essential to the signal transduction process in cells, and link ligand-activated integral membrane receptors to complex intracellular biochemical pathways. Less-well studied is the role of these molecules in downregulating signaling events. Our studies focus on the fate of the epidermal growth factor receptor (EGFR) and the Grb2 adaptor molecule upon activation of the receptor at the cell surface, and address questions about the function of Grb2 in EGFR dynamics.

Grb2 consists of an SH2 domain flanked by two SH3 domains (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992). The SH2 domain, by its interactions with phosphotyrosine residues in a specific sequence context, mediates binding to a variety of activated receptors and adaptor molecules. The SH3 domains of Grb2 bind proline-rich sequences such as those found in the Ras guanine nucleotide exchange factor, SOS (Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). Hence, Grb2 links ligand-activated receptors coupled to tyrosine kinases to the distal signaling apparatus.

The EGFR has been extensively investigated over many years, and it serves as an excellent model of a receptor that mediates such diverse cellular phenomena as proliferation and differentiation (Sorkin, 1998). The EGF ligand induces dimerization of the receptor, which leads to activation of the protein tyrosine kinase activity intrinsic to the receptor. Phosphorylation of multiple tyrosine residues on the cytosolic domain of the receptor creates binding sites for interaction with enzymes such as phospholipase Cγ1 [PLCγ1 (Zhu et al., 1992)] and phosphatidylinositol 3-kinase [PI3K (Hu et al., 1992)], and the adaptors Shc (Okabayashi et al., 1994; Soler et al., 1994) and Grb2 (Buday and Downward, 1993; Lowenstein et al., 1992; Okutani et al., 1994). Such interactions are critical to the induction of those signaling events that the receptor regulates.

Attenuation of EGFR signals has also been extensively studied. One mechanism for downregulation is EGFR internalization. It has been reported that mutant EGFR receptors that are defective in internalization lead to ligand-induced transformation (Wells et al., 1990), supporting the importance of receptor downregulation. To date, the role for Grb2 in EGFR internalization has been controversial, and biochemical and immunofluorescence microscopy studies have produced conflicting results (Chang et al., 1991; Chang et al., 1993;
Wang and Moran, 1996). The exact function of Grb2 in EGF receptor dynamics remains poorly understood.

In this study we made use of the GFP spectral variants, YFP and CFP (Ellenberg et al., 1998; Ellenberg et al., 1999), which we fused to Grb2 and EGFR, respectively. Because YFP and CFP are distinguishable from each other based on the difference in their fluorescence excitation and emission patterns, we were able to follow the spatio-temporal relationship of these two chimeric molecules (Grb2-YFP and EGFR-CFP) in single live cells using confocal microscopy. We show that Grb2-YFP binds EGFR-CFP in an SH2-dependent fashion. Following activation, both internalize together in large macropinocytic structures that are temporally, morphologically and biochemically distinct from conventional transferrin-containing endocytic structures. Expression of Grb2-YFP containing mutations in either SH3 domain did not block recruitment of Grb2 to the plasma membrane upon EGF stimulation but impeded the subsequent inward translocation of the macropinocytic structures enriched in Grb2 and EGFR. These results demonstrate a role of Grb2 in events associated with EGFR internalization in activated cells.

Materials and Methods

Reagents

Reagents used in this article were purchased as indicated: murine EGF (Calbiochem); mouse anti-EGF, anti-Grb2, anti-SOS1/2, anti-Cbl and anti-dynamin II antibody (Santa Cruz Biotechnology); sheep anti-EGFR antibody (Upstate Biotechnology); anti-GFP antibody (monoclonal and polyclonal; Clontech); TRITC-labeled rabbit anti-goat IgG and goat anti-mouse IgG antibody (Kirkegarrd & Perry Laboratories Inc.); biotin-XX EGF conjugate (B-EGF) (Molecular Probes); streptavidin-Quantum Red conjugate (Sigma).

Cells

Both A431 cells and COS-7 cells were grown in complete DMEM medium (DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 50 µg/ml gentamicin).

Constructs

Both YFP and CFP (human codon-optimized W7) GFP variants were described previously (Ellenberg et al., 1998; Ellenberg et al., 1999). pYFP-N1 and pCFP-N1 vectors have the same backbone as pEGFP-N1 and pCFP (Ellenberg et al., 1998; Ellenberg et al., 1999), which described previously (Ellenberg et al., 1998; Ellenberg et al., 1999). Both YFP and CFP (Ellenberg et al., 1998; Ellenberg et al., 1999), which

Plasmid constructions were conducted as follows: (1) pYFP/Grb2. The BamHI-EcoRI fragment from the pGEX-3X plasmid containing human Grb2 was ligated to the BamHI-EcoRI fragment of pBluescript II KS (pBKS/Grb2). The AflHI-NspI fragment was removed from pBKS/Grb2, and then the vector was religated using the oligos 5′ TTT CCC CCC GAT TAT GTC ACC CCC GTG AAC CGG AAG GTC GGT GGA GGT GGA GCC GTA 3′ and 5′ CAT GTA CCG CTC CAC CGA CGT TCC GGT TCA CGG GGG TGA CAT AAC GAA ACA TG 3′ (pBKS/Grb2-4G). The SalI-AgelI fragment from pBKS/Grb2-4G was ligated to SalI-AgelI-digested pYFP-N1 vector (pYFP/Grb2).

(2) pYFP/R86K Grb2 (encoding SH2m-YFP). The BamHI-BglII fragment from the pGEX-3X plasmid containing human Grb2 with a R86K mutation was ligated to the BglII-digested pYFP/Grb2 (pYFP/R86K Grb2).

(3) pYFP/P49L Grb2 (encoding NSH3m-YFP). The BamHI-BglII fragment from the pGEX-3X plasmid containing human Grb2 with a P49L mutation was ligated to the BglII-digested pYFP/Grb2 (pYFP/P49L Grb2).

(4) pYFP/P49L, G203R Grb2 (encoding NCSH3m-YFP). A PCR product with the G203R mutation was created using pGEX-3X plasmid containing G203R Grb2 as a template with the oligos 5′ TAT CAC AGA TCT ACA TCT 3′ and 5′ GCC GAC CGG TCC ACC GAC GTT CCG GTT CAG GGC TGG 3′. Then, the BglII-AgelI-digested PCR product was ligated to the BglII-AgelI fragment of pYFP/P49L Grb2 (pYFP/P49L, G203R Grb2). The sequence of this construct was verified using the Sequenase II DNA sequencing kit.

(5) pYFP/P49L Grb2 (encoding CSH3m-YFP). The Nhel-BglII fragment of pYFP/P49L, G203R Grb2 was replaced with XbaI-BglII fragment from pYFP/Grb2 to remove the P49L mutation (pYFP/P49L Grb2).

(6) pCFP/EGFR (encoding EGFR-CFP). The XhoI fragment from LTR2-EGFR (kindly provided by J. Rubin, Laboratory of Cellular and Molecular Biology, NCI, NIH) containing the entire human EGFR was ligated to XhoI-digested pBluescript II KS (pBKS-EGFR). The HindIII-KpnI fragment from pBKS/EGFR was subcloned into HindIII/KpnI-digested pCFP-N1 vector (pCFP/EGFR Hind-Kpn). pCFP/EGFR Hind-Kpn was digested with SalI, filled in and cloned into pBKS, and then religated (pCFP/Sal BL). The junction between the EGFR and CFP was created by PCR using pBKS-EGFR as a template together with the oligos 5′ GCC ACC CAT ATG TAC CAT C 3′ and 5′ GCC CCG CGG TGC TCC AAC AAA TTC ACT G 3′. The Accl/SacII-digested PCR product was ligated to the Accl-SacII fragment of pCFP/Sal BL (pCFP/EGFR).

Transfection

COS-7 cells were transfected by electroporation using 10 µg of pCFP/EGFR and 5 µg of either pYFP/Grb2 or pYFP/mutant Grb2. 10 µg of either pYFP/Grb2 or pYFP/mutant was transfected into A431 cells. Electroporation was performed at 250 V and 500 µF for COS-7 cells and at 310 V and 960 µF for A431 cells with a Gene Pulser (Bio-Rad).

Confocal time-lapse imaging

Transfected cells were grown in coverglass chambers (LabTek) for 24 hours in complete DMEM medium. The medium was then replaced with DMEM medium free of phenol red and supplemented with 0.5% FBS, 2 mM glucose and 50 µg/mg gentamicin. After a further 16 hours of incubation, the cells were subjected to time-lapse imaging. For dual color GFP time-lapse imaging (Ellenberg et al., 1998; Ellenberg et al., 1999), the chambers were mounted on a temperature-controlled stage of a confocal microscope (model LSM 410; Carl Zeiss) using the 63× objective (N.A. 1.4). To image YFP, the 488 nm laser line (Omnichrome Series 43, Omnichrome Inc.) was used with a standard dual FT 488/568 dichroic and a BP 515-560 (both Carl Zeiss). To image CFP, the 413 nm Kr laser line (Coherent Enterprise II, Coherent Inc.) was used in conjunction with a FT440 dichroic and a LP 460 emission filter (both Chroma Technology Corp.). Images of Grb2-YFP and EGFR-CFP were captured just before addition of EGF (100 ng/ml for COS-7 and 500 ng/ml for A431, Calbiochem) directly to the chamber, and subsequent images were taken every 30 seconds. Time-lapse sequence was recorded using a macro program that allows autofocusing on the coverslip surface in the reflection mode before capturing confocal fluorescence images.

Immunofluorescence staining of fixed cells

COS-7 cells were grown in coverglass chambers (LabTek) for 24
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hours in complete DMEM medium, which was then replaced with DMEM medium free of phenol red supplemented with 0.5% FBS, 2 mM glutamine, and 50 µg/mg gentamicin. After a further 16 hours incubation, EGF stimulation (100 ng/ml) was carried out. Cells were then fixed in 3.7% paraformaldehyde in PBS for 30 minutes at room temperature, washed (three times) in PBS containing 10% fetal bovine serum (PBS/FBS), permeabilized using 0.1% Triton X-100 in PBS for 4 minutes at room temperature, washed (three times), and incubated for 45 minutes in PBS/FBS for preblocking. Cells were then incubated with anti-EGFR antibody in PBS/FBS for 45 minutes at room temperature, washed, and incubated with TRITC-labeled goat anti-mouse IgG antibody for 45 minutes, followed by washing with PBS (three times). Stained samples were viewed using the 568-nm laser line of a confocal laser scanning microscope using the 63· objective (NA 1.4).

Uptake assays

Transfected cells were incubated as described above in complete DMEM medium, then in DMEM medium containing 0.5% FBS before assays. Cells were incubated with transferrin-TRITC conjugate (50 µg/ml) (Molecular Probes) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging. For uptake assays in the presence of EGF, cells were incubated with transferrin-TRITC at 4°C for 40 minutes, washed with ice-cold phenol-red-free DMEM containing 0.5% FBS, then incubated with the same medium containing EGF (100 ng/ml) at 37°C for 40 minutes. Following three washes with phenol-red-free DMEM medium containing 0.5% FBS, uptake of transferrin-TRITC was monitored in the absence of EGF by time-lapse imaging.
2×10⁶ cells/ml in DMEM containing 0.5% BSA and 25 mM Hepes (buffer A) at 0°C. Either B-EGF or B-Tfn was added to a final concentration of 40 ng/ml or 10 μg/ml, respectively, and incubation was continued for 15 minutes at 0°C. Unbound B-EGF or B-Tfn was removed by washing with buffer A at 0°C. Cells were split into aliquots (2×10⁵ cells), incubated at 37°C for the indicated times and then returned to icy water. 5 μl of streptavidin-Quantum Red was added to 20 μl aliquots of cells resuspended in PBS containing 0.1% BSA and 0.1% sodium azide (staining buffer). Following washing with staining buffer, data was collected on a FACScan and analyzed using CELLQuest software (Becton-Dickinson). Avidin inaccessibility (AI) was calculated as in the equation,

$$ AI(\%) = \left[ 1 - \frac{(MF_t - BG)}{(MF_0 - BG)} \right] \times 100, $$

where MFₜ is the mean fluorescence intensity of cells stimulated for the indicated times (t), MF₀ is the mean fluorescence intensity of unstimulated cells (time 0), and BG is the mean fluorescence intensity of cells incubated at 0°C with streptavidin-Quantum Red alone.

To determine the effect of EGF stimulation on Tfn uptake, avidin inaccessibility was similarly measured except that cells were incubated with B-Tfn (10 μg/ml) plus EGF (100 ng/ml). Non-specific binding of either B-EGF or B-Tfn was determined in the presence of at least 100-fold molar excess unlabeled EGF or Tfn, respectively.

Immunoprecipitation assays
Unstimulated or EGF-stimulated transfected cells were lysed in Brij/Octyl glucoside lysis buffer (1% Brij 97, 0.5% octyl glucoside, 150 mM NaCl, 5 mM EDTA) on ice for 30 minutes. After centrifugation, the supernatant was collected. Lysates were subjected to immunoprecipitation with an anti-GFP antibody. Anti-GFP immunoprecipitates were then analyzed by SDS-PAGE and western blotting using relevant antibodies.

Fig. 2. EGF-induced redistribution of Grb2-YFP to the cell periphery in A431 cells. A431 cells were transfected with YFP fused to either wild-type Grb2, the SH2 domain mutant or the SH3 domain mutants of Grb2, as follows. (A,B) Wild-type Grb2-YFP, (C,D) SH2m-YFP, (E,F) NSH3m-YFP, (G,H) CSH3m-YFP and (I,J) NCSH3m-YFP. Images of live A431 cells in each column were taken prior to (A,C,E,G,I) and 1 minute after EGF stimulation (B,D,F,H,J) using confocal microscopy. Bars, 5 μm.

Fig. 3. Internalization of EGFR-CFP with Grb2-YFP induced by EGF. Individual live COS-7 cells co-expressing wild-type Grb2-YFP and EGFR-CFP were monitored over time by confocal time-lapse imaging. Protein movement of Grb2-YFP (middle) and EGFR-CFP (left) upon EGF stimulation is shown. Arrows point to the characteristic internalized structures (see text).
Grb2 is necessary for EGF-stimulated EGFR uptake

Results

GFP chimeric proteins that contain Grb2 or EGFR

Extensive biochemical evidence demonstrates that Grb2 has a cytosolic distribution in resting cells. Upon activation of certain receptor tyrosine kinases or ligation of receptors that are coupled to tyrosine kinases, Grb2 translocates to the plasma membrane to bind target molecules. The fate of Grb2 following these events is less well characterized. In order to follow this translocation visually and to observe the entire course of Grb2 molecular dynamics, we created Grb2-YFP and EGFR-CFP.

In addition to preparing a chimera of wild-type Grb2 with YFP (Grb2-YFP, Fig. 1A), we also added YFP to the C-terminus of several Grb2 mutants (SH2m-, NSH3m-, CSH3m- and NC5H3m-YFP) in order to investigate the contribution of the individual SH2 and SH3 domains to the dynamics of Grb2. The R86K (arginine 86 to lysine) mutation in the SH2 domain is known to disrupt SH2 domain-phosphotyrosine interactions (Clark et al., 1992). The mutations P49L (proline 49 to leucine) and G203R (glycine 203 to arginine) are in the N- and C-terminal SH3 domains, respectively. These mutants of Grb2 correspond to loss-of-function phenotypes in Caenorhabditis elegans (Clark et al., 1992) and are known to block SH3-mediated interactions (Egan et al., 1993). For all Grb2-YFP fusion proteins, four glycine residues were placed as a linker between Grb2 and YFP to increase flexibility in that region.

We fused CFP to the EGFR C-terminus (EGFR-CFP, Fig. 1B). A similar chimera, where EGFR was tagged with the enhanced GFP (EGFP) at the C-terminus, was already reported to behave like endogenous EGFR, exhibiting such expected function as tyrosine phosphorylation upon EGF stimulation, association with Shc and endocytosis via an interaction with AP-2 (Carter and Sorkin, 1998). All GFP chimeric molecules in this study demonstrated the expected apparent molecular weight by western blotting using an anti-GFP antibody (52 kDa for Grb2-YFP and 207 kDa for EGFR-CFP), and were detected specifically with antibodies directed against Grb2 or EGFR (Fig. 1C,D).

Wild-type Grb2 tagged with YFP functions similarly to Grb2

Grb2 is known to associate with a variety of receptors upon receptor ligation via interactions of its SH2 domain and phosphotyrosine residues. In addition, the SH3 domains of Grb2 mediate Grb2 binding to critical signaling molecules such as SOS (Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993), Cbl (Buday et al., 1996; Hanazono et al., 1996; Meisner et al., 1995), WASP (Cory et al., 1996; She et al., 1997), MEKK1 (Pomerance et al., 1998) and dynamin (Ando et al., 1994; Barylko et al., 1998; Gout et al., 1996; Herskovits et al., 1993; Lin et al., 1997; Wang and Moran, 1996).

To test whether Grb2-YFP behaves like Grb2, we performed immunoprecipitation assays using cell lysates from A431 cells transfected with wild-type Grb2-YFP. EGF was co-immunoprecipitated with Grb2-YFP upon EGF stimulation (Fig. 1E), suggesting that Grb2-YFP becomes inducibly bound to EGFR as does endogenous Grb2. Additionally, anti-GFP immunoprecipitates from A431 cells expressing wild-type Grb2-YFP (Fig. 1F, lane 2) contained SOS, Cbl and dynamin without EGF stimulation, showing the constitutive associations of Grb2-YFP with these critical molecules. Based on its binding specificity, Grb2-YFP behaves similarly to Grb2.

SH2-domain-dependent translocation of Grb2-YFP to the plasma membrane

To demonstrate by imaging that EGF stimulation induces the redistribution of Grb2 to the plasma membrane, we expressed Grb2-YFP, as well as Grb2 mutant-YFP fusion proteins in A431 cells. A431 human epidermoid cells were used as they bear abundant EGFR (Gamou et al., 1984; Haigler et al., 1979; Krupp et al., 1982). Grb2-YFP was diffusely cytosolic in the resting state (Fig. 2A). Translocation towards the cell periphery began immediately after EGF was added. Redistribution of Grb2-YFP to the plasma membrane was completed within a minute of EGF stimulation (Fig. 2B). Concurrently, the amount
of Grb2-YFP in the cytoplasm decreased 1 minute after EGF stimulation, leaving a central lucency. We also tested whether an N-terminally tagged Grb2 construct, YFP-Grb2, redistributed to the plasma membrane in the same fashion. YFP-Grb2 translocation upon EGF stimulation was almost identical to C-terminally tagged Grb2 (data not shown), indicating that the redistribution of the Grb2 chimeras with GFP can be attributed to the Grb2 sequence itself. These observations support the biochemical evidence for EGF-induced translocation of Grb2 from the cytosol to the plasma membrane where Grb2-EGFR interactions occur. Note that a significant amount of Grb2 accumulates in the nucleus after EGF stimulation as previously reported (Romero et al., 1998).

We expressed SH2m-YFP in A431 cells to confirm that translocation to the membrane was dependent on the SH2 domain interactions with phosphorylated EGFR. As expected, no redistribution of SH2m-YFP was seen upon EGF stimulation (Fig. 2C,D), indicating that translocation of YFP-tagged Grb2 to the plasma membrane was dependent on an intact Grb2 SH2 domain. Grb2 SH3 domain mutants tagged with YFP (NSH3m-, CSH3m- and NCSH3m-YFP) translocated to the plasma membrane with similar kinetics to that of wild-type Grb2-YFP in response to EGF (Fig. 2F,H,J). Thus, EGF-induced redistribution of Grb2-YFP to the plasma membrane was not only SH2 domain dependent, but did not require intact SH3 domains.

EGFR-CFP recruits Grb2-YFP to the plasma membrane and is internalized with Grb2-YFP

To address the spatio-temporal relationship between Grb2 and EGFR upon receptor activation with EGF, we co-expressed both wild-type Grb2-YFP (Grb2-YFP) and EGFR-CFP. To avoid any complication because of the large amount of endogenous EGFR on A431 cells (1-2×10⁶/cell) (Gamou et al., 1984; Haigler et al., 1979; Krupp et al., 1982), Grb2-YFP and EGFR-CFP were co-expressed in COS-7 cells, which have low levels of endogenous EGFR. In unstimulated cells, EGFR-CFP localized both to the plasma membrane and to punctate structures scattered in the periphery and the perinuclear region, whereas Grb2-YFP was diffusely cytosolic (Fig. 3, pre-EGF stimulation). A significant pool of Grb2-YFP redistributed to the plasma membrane within a minute of EGF stimulation (Fig. 3, 1 minute), where it co-localized with EGFR-CFP. Note also that Grb2-YFP also accumulated transiently in the nucleus, as found in A431 cells. EGF-induced redistribution of Grb2-YFP in COS-7 did not occur unless EGFR-CFP was co-expressed (data not shown), which suggested that the phosphorylation of endogenous EGFR was not sufficient to make translocation of Grb2-YFP detectable in this context. Importantly, Grb2-YFP was not recruited to the internal structures containing EGFR-CFP upon EGF stimulation (Fig. 3, 1 minute and 4 minute). Given that redistribution of Grb2-YFP to the plasma membrane was clearly observed upon EGF stimulation either in A431 cells expressing large amounts of endogenous EGFR, or in COS-7 cells co-expressing EGFR-CFP, it appears that recruitment of Grb2-YFP to the plasma membrane in EGF-stimulated cells is mediated by EGFR.

Immediately following Grb2-YFP translocation to the plasma membrane upon EGF stimulation, the cells began to exhibit ruffling activity at their plasma membrane (Fig. 3). Spherical and sack-like structures reminiscent of macropinosomes that contained Grb2-YFP and EGFR-CFP then started to appear at and detach from the plasma membrane.
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The accompanying movie shows high resolution images and video sequences of these structures, which moved towards the perinuclear region over time with both Grb2-YFP and EGFR-CFP remaining associated (see http://jcs.biologists.org/supplemental). The perinuclear pool of EGFR-CFP observed prior to activation remained distinct from the large macropincytotic structures containing Grb2-YFP and EGFR-CFP, indicating that only Grb2-YFP was recruited to and remained associated with membranes containing activated EGFR.

Grb2-YFP-containing structures that internalize in response to EGF contain dynamin but not clathrin, transferrin or AP2

To further characterize the large spherical structures containing Grb2-YFP and EGFR-CFP that arose off the plasma membrane during EGF stimulation, we performed double-labeling experiments using antibodies to proteins known to participate in clathrin-mediated receptor internalization, including clathrin, AP2 and dynamin (Schmid et al., 1998) (Fig. 4). We also compared the distribution of the spherical structures to that of Tf, which is internalized into the clathrin-mediated endocytic pathway (Fig. 5A, top row). When we stained cells expressing Grb2-YFP with antibodies to clathrin heavy chain (CHC), which assembles with clathrin light chain to form clathrin lattices on the cytosolic surfaces of membranes (Marsh and McMahon, 1999), very little, if any, labeling of the Grb2-YFP-containing spherical structures was observed (Fig. 4, CHC). Antibody staining of the adaptor protein AP2, which helps recruit cargo into clathrin-coated pits (Marsh and McMahon, 1999), revealed that this protein also was not enriched on the Grb2-YFP-containing structures (Fig. 4, AP-2). However, significant labeling of the Grb2-YFP-containing structures with dynamin antibodies was observed (Fig. 4, Dynamin). TRITC-Transferrin, used to monitor clathrin-mediated endocytosis, showed a pattern of uptake that was similar to that observed in unstimulated cells and that did not co-localize with Grb2-YFP (Fig. 5A). Moreover, the rate of uptake of biotinylated transferrin (Tfn) in the activated cells was indistinguishable from unstimulated cells (Fig. 5B). These data suggest that EGF-induced, Grb2-YFP-containing structures are distinct from conventional clathrin-derived endocytic intermediates, and internalize plasma membrane receptors, such as EGFR, in a pathway that operates in parallel to the clathrin-mediated uptake pathway.

Fig. 6. Internalization of EGFR-CFP is not blocked by the inhibition of clathrin-mediated uptake. COS-7 cells were co-transfected with EGFR-CFP and the C-terminal domain of AP180 and serum starved. In nontransfected cells, transferrin localizes to the perinuclear region. In co-transfected cells, the C-terminal domain of AP180 inhibits clathrin-mediated uptake and transferrin fails to localize (left). When stimulated with EGF, co-transfected cells still take up EGFR-CFP in large structures (right). Images from a time-lapse series are presented. At time 0, EGF was added to a final concentration of 100 ng/ml. Confocal images were captured every 30 seconds. Arrows indicated a region of interest initially on the plasma membrane. The region is internalized and moves to the perinuclear region where it deforms the nuclear envelope.
Inhibition of clathrin-mediated uptake by AP-180 overexpression does not prevent EGFR internalization in response to EGF stimulation of starved cells

To rule out a role of clathrin in the uptake of EGFR-CFP in response to EGF, we co-expressed EGFR-CFP and the C-terminal domain of AP180 in COS cells. AP180 is an adaptor protein that plays an important role in clathrin-mediated endocytosis (Ford et al., 2001). When overexpressed in cells, it has been shown to block clathrin-mediated uptake of proteins from the plasma membrane (Ford et al., 2001). To assess the extent of inhibition of the clathrin-mediated pathway by AP180 overexpression, transfected cells were incubated with rhodamine-labeled transferrin. As shown in Fig. 6, transferrin failed to localize to the perinuclear region in cells overexpressing AP180. When serum-starved cells were stimulated with EGF, EGFR-CFP still internalized into large macropinocytic structures in cells overexpressing AP180. The data thus indicate that EGFR internalization into macropinocytic structures in response to EGF after serum starvation is not mediated by clathrin.

Grb2 SH3 domain mutants prevent inward translocation of endocytic structures that have sequestered EGF receptors in EGF-stimulated cells

We next sought to clarify the role of Grb2 in the events associated with the EGF-stimulated EGFR uptake pathway. We co-expressed EGFR-CFP in COS-7 cells with SH3 mutants of Grb2 to test whether disruption of the structural integrity of Grb2 SH3 domains affected the dynamics of EGFR internalization. Prior to EGF stimulation, cells expressing an NSH3 mutant of Grb2 tagged with YFP (NSH3m-YFP) showed that the chimera was diffusely cytosolic, whereas EGFR-CFP was localized on the plasma membrane and in perinuclear structures (Fig. 7, 0'). Within 1 minute of EGF stimulation, NSH3m-YFP molecules translocated to the plasma membrane (Fig. 7, 1'), similar to that observed for Grb2-YFP molecules shown above. However, the subsequent fate of NSH3m-YFP and EGFR-CFP at the plasma membrane was different from that observed for wild-type Grb2-YFP and EGFR-CFP (Fig. 7, 20', 30'). NSH3m-YFP and EGFR-CFP remained peripherally localized instead of moving into the cytoplasm (Fig. 7). They remained closely apposed to the plasma membrane for significant periods of time after addition of EGF, with the large spherical structures containing NSH3m-YFP and EGFR-CFP unable to translocate inwards. These structures were relatively static and often appeared in confocal sections to be tethered by thin membrane tubules with the plasma membrane (Fig. 7, inset). A blockade of the inward translocation of peripheral structures containing EGFR-CFP was similarly observed in EGF-activated cells overexpressing either CSH3m- or NCSH3m-YFP. Movie versions of the blockade induced by NSH3m-, CSH3m- or NCSH3m-YFP can be found at http://jcs.biologists.org/supplemental.

The EGF-induced, spherical structures containing EGFR-CFP in NSH3m-YFP-expressing cells could be labeled with dynamin antibodies, but not with antibodies to CHC or AP-2 (Fig. 8). Additionally, TRITC-Tfn that was
Grb2 is necessary for EGF-stimulated EGFR uptake

Fig. 8. Colocalization of NSH3m-YFP with clathrin heavy chain, AP-2 and dynamin. COS-7 cells co-expressing EGF-R-CFP with NSH3m-YFP were stimulated. Cells were then immunostained with antibodies directed at clathrin heavy chain (CHC), AP-2 or dynamin, followed by a TRITC-conjugated secondary antibody. Arrows point to co-localized structures.

Confocal images in the Z-axis showed that they were always closely associated with the plasma membrane (data not shown). By contrast, without permeabilization, these structures (Fig. 9C, arrows) could not be labeled with the antibody (Fig. 9D), indicating that EGFRs in them were not accessible to this antibody. This suggested that, in the presence of Grb2 SH3 mutants, EGFR and Grb2 are sequestered into domains of the plasma membrane that are no longer exposed to the outside. Consistent with this, we found that biotinylated EGF could be internalized into these cells (measured by avidin inaccessibility) at a rate similar to the rate in cells expressing wild-type Grb2-YFP (Fig. 9E). These results indicate that, in cells expressing Grb2 SH3 domain mutants, EGF-stimulation results in uptake of EGFR into endocytic structures that remain closely associated with the plasma membrane and that are inaccessible to the extracellular environment.

Discussion

The adaptor protein Grb2 has been extensively analyzed biochemically and is known to link surface receptors to downstream signaling molecules. Upon receptor stimulation, Grb2 translocates from the cytosol to the cell surface where it binds, via its SH2 domain, receptors at specific sites of tyrosine phosphorylation. Once bound to receptors, Grb2 is thought to use its SH3 domains to link ligand-activated receptors to the distal signaling apparatus. Whether Grb2 plays an additional role in receptor internalization, which attenuates receptor signaling, has been controversial.

For the EGFR, which is known to interact with Grb2, early reports suggested that Grb2 might not regulate EGFR internalization, since internalization of EGFR mutants lacking the Grb2-binding domain still occurred but at a slower rate than normal (Chang et al., 1991; Chang et al., 1993). However, Wang and Moran showed that EGF-induced EGFR endocytosis was blocked in MDCK cells following microinjection of Grb2 containing the G203R C-SH3 domain mutation (Wang and Moran, 1996), suggesting that Grb2 functions in EGF-induced EGFR endocytosis. Further support to this view was provided by Sorkin et al., who showed that in EGF-stimulated cells EGFR-CFP and Grb2-YFP co-localize in endosomes and can undergo fluorescence energy transfer (FRET), an indicator of protein-protein interactions (Sorkin et al., 2000). However, the specific role Grb2 plays in EGF-stimulated EGFR internalization (e.g. receptor sorting, vesicle budding/pinching or vesicle transport) has remained unclear.

In the present study we clarify the role of Grb2 in EGF internalization by directly visualizing EGFR and Grb2

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EGF receptors internalized in EGF-stimulated cells expressing Grb2 SH3 domain mutants are inaccessible to the cell exterior

To further investigate the nature of the inhibitory effect on EGF-stimulated EGFR trafficking caused by expression of Grb2 SH3 domain mutants, we asked whether the spherical structures containing EGFR-CFP that localized near the plasma membrane under these conditions represented fully budded membrane vesicles whose contents were inaccessible to the cell exterior, or represented vesicles still continuous with the plasma membrane whose contents were accessible to the extracellular space. To distinguish between these possibilities, EGF-stimulated COS-7 cells expressing EGFR-CFP and NSH3m-YFP were either permeabilized or left unpermeabilized following fixation, and were then immunostained with an antibody directed against the extracellular domain of EGFR. In permeabilized COS-7 cells, most of the spherical structures associated with the plasma membrane were detected by the antibody (Fig. 9B). Some of the spherical structures appeared to be detached from the plasma membrane, but 3D reconstruction based on a series of
dynamics in EGF-stimulated cells expressing wild-type or mutant SH2/SH3 domains of Grb2. Grb2-YFP redistributed to the cell periphery immediately after EGF stimulation. This redistribution required an intact Grb2 SH2 domain consistent with previous biochemical data (Lowenstein et al., 1992). Mutation of either or both Grb2 SH3 domains had no effect on the dynamics of translocation to the plasma membrane. However, time-lapse confocal microscopy revealed that the SH3 mutants prevented the stimulation-dependent inward translocation of EGF-containing endocytic structures that was observed in wild-type Grb2-expressing cells. Therefore, the SH3 domains of Grb2 appear to orchestrate the machinery necessary for translocating endocytic membranes carrying EGFR from the periphery to the interior of the cell.

Several properties of the endocytic membranes carrying EGFR-CFP and Grb2-YFP in EGF-stimulated cells suggested that they were distinct from clathrin-derived endocytic structures. First, their appearance was coupled to the translocation of Grb2-YFP to the plasma membrane and the subsequent ruffling activity of these membranes, which often seemed to give rise directly to the large, EGF-CFP- and Grb2-YFP-containing endosomes. Second, the surfaces of these endocytic membranes were depleted of molecules associated with clathrin-coated pits, including clathrin heavy chain and AP-2 (Marsh and McMahon, 1999). Third, they did not contain internalized transferrin, which is a marker for the clathrin-derived endocytic system. Finally, the macropinocytic structures carrying EGFR-CFP into the cell were not inhibited when clathrin-mediated uptake was blocked by overexpression of AP180, a clathrin adaptor molecule. Because we did not characterize the fate of EGF-CFP and Grb2-YFP for periods after EGF stimulation longer than 60 minutes, it is possible that these molecules eventually are delivered into the conventional endocytic pathway leading to lysosomes. Our time-lapse images early after stimulation with EGF, however, demonstrate that EGFR-CFP and Grb2-YFP at the plasma membrane are internalized into large structures that do not resemble in size or composition the endocytic structures derived from clathrin-coated pits.

Insight into the role of Grb2 in the nonclathrin uptake pathway followed by EGF-CFP and Grb2-YFP was gained from experiments expressing GFP-tagged Grb2 mutants where the structural integrity of the Grb2 SH3 domain was disrupted. When these mutants were expressed in cells, they translocated normally to the plasma membrane in response to EGF stimulation and then clustered with EGF-CFP within large, endocytic structures. However, these structures did not subsequently translocate into the interior of the cell, in contrast to what is

![Fig. 9. EGFR internalization is blocked by NSH3m-YFP at the step of membrane fission. EGF-stimulated COS-7 cells co-expressing NSH3m-YFP and EGFR-CFP were fixed then either permeabilized (A,B) or left unpermeabilized (C,D). Cells were immunostained with an antibody against the extracellular domain of EGFR, followed by a TRITC-conjugated secondary antibody (B,D). Bar, 5 μm. (E) EGFR internalization in COS-7 cells overexpressing the indicated YFP chimeric molecules was measured by determining avidin inaccessibility. Assays were performed as described in Materials and Methods. Data shown are representative of three independent experiments.](image-url)
observed in wild-type Grb2-expressing cells. Thus, the SH3 domains of Grb2 are necessary for inward translocation of Grb2- and EGFR-containing endocytic structures, but not for stimulus-induced Grb2 recruitment to membranes and the clustering/uptake of EGFR into large endocytic structures. Despite the inhibition of movement of EGFR-containing endocytic structures by the Grb2 SH3 domain mutants, TfR was internalized normally with or without EGFR stimulation in the presence of these Grb2 mutations. This finding supports the idea that the endocytosis machinery for EGFR differs from that used by the TfR, consistent with the previous evidence (Warren et al., 1997; Warren et al., 1998).

Flow cytometric analysis measuring avidin inaccessibility showed that, in the presence of Grb2 SH3 mutations, EGFR-induced membrane vesicles could sequester internalized contents, but the vesicles were unable to detach from the plasma membrane and move into the cytoplasm. Therefore, an important focus of future work will be in determining which binding partner(s) of the Grb2 SH3 domain and their downstream signaling pathways are involved in vesicle release and translocation into the cell center. Dynamin is thought to mediate the process of pinching off vesicles from the membrane (Schmid et al., 1998). In addition, the GTPase activity of dynamin is reported to be enhanced by its interaction with the Grb2 SH3 domains (Barylko et al., 1998; Gout et al., 1993; Herskovits et al., 1993). Therefore, disruption of dynamin-Grb2 interactions by mutations in the Grb2 SH3 domains might be predicted to lead to the failure in targeting of dynamin to the cell periphery, explaining the inhibition of vesicle release. Unexpectedly, however, dynamin was found to redistribute to the cell periphery upon EGFR stimulation regardless of the presence of the Grb2 SH3 domain mutants (data not shown). Although it is possible that the redistributed dynamin GTPase activity was not enhanced sufficiently in the presence of these mutants, it seems more likely that some other protein binding to the Grb2 SH3 domains is responsible for the release of these vesicles from the plasma membrane and their ability to translocate inward to the cell center.

In summary, we have examined the dynamics of Grb2 and EGFR fused to GFP spectral variants in single live cells, and have demonstrated that interactions through Grb2 SH3 domains regulate EGFR internalization. We further show that Grb2 is responsible for coupling EGFR-containing membranes with downstream effectors involved in the internalization of these membranes through a macropinocytic pathway. Grb2 has previously been thought to serve solely as an adapter linking receptors on the plasma membrane to downstream signaling pathways. Our results shed light on the additional function of Grb2, which has previously been thought to serve solely as an adapter linking receptors on the plasma membrane through a macropinocytic pathway. Grb2 has been shown to regulate the activity of dynamin, a GTPase that is involved in the formation of endocytic vesicles. Grb2 binds to dynamin and enhances its GTPase activity, which is thought to be necessary for vesicle formation.

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