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Ligand-induced clathrin-mediated endocytosis of the keratinocyte growth factor receptor occurs independently of either phosphorylation or recruitment of eps15

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Abstract Keratinocyte growth factor receptor (KGFR) is a receptor tyrosine kinase expressed on epithelial cells. Following ligand binding, KGFR is rapidly activated and internalized by clathrin-mediated endocytosis. Among the possible receptor substrates which could be involved in the regulation of KGFR endocytosis and down-modulation, we analyzed here the eps15 protein in view of the proposed general role of eps15 in regulating clathrin-mediated endocytosis as well as that of eps15 tyrosine phosphorylation in the control of regulated endocytosis. Immunoprecipitation and Western blot analysis showed that activated KGFR was not able to phosphorylate eps15, suggesting that eps15 is not a receptor substrate. Double immunofluorescence and confocal microscopy revealed that activated KGFR, differently from epidermal growth factor receptor (EGFR), did not induce recruitment of eps15 to the cell plasma membrane. Microinjection of a monoclonal antibody directed against the C-terminal DPF domain which contains the AP2 binding region of eps15 led to inhibition of both pathways of receptor-mediated endocytosis, the EGFR ligand-induced endocytosis and the transferrin constitutive endocytosis, but did not appear to block the KGFR ligand-induced internalization. Taken together our results indicate that the clathrin-mediated uptake of KGFR is not mediated by eps15.

Key words: Keratinocyte growth factor receptor; Epidermal growth factor receptor; Eps15; Endocytosis

1. Introduction

Clathrin-mediated endocytosis represents a selective and efficient cellular mechanism of uptake, regulated by multiple interactions among proteins which function as adapters or accessory components and form active complexes. Among them, the eps15 protein was initially identified as a substrate of the epidermal growth factor receptor (EGFR) [1], but several further lines of evidence strongly suggested that eps15 is involved in receptor-mediated endocytosis. The eps15 protein, in fact, is constitutively associated with the adapter protein AP2 [2] and contains three eps15 homology domains in the N-terminal portion, responsible for multiple interactions with proteins of the endocytic machinery [3–5]. Moreover, the inhibition of both EGFR and transferrin receptor (TfR) internalization through microinjection of anti-eps15 antibodies or overexpression of dominant negative mutants of eps15 suggested that the protein could play a general role in the control of either constitutive or ligand-induced endocytosis [6,7]. Previous observations from our group, in addition, showed that eps15 colocalizes with EGFR during early and late steps of endocytosis, suggesting that the protein could have multiple roles in different stages of the endocytic process [8]. Finally, it has been proposed that eps15 phosphorylation is essential for ligand-induced EGFR, but not for constitutive TfR internalization [9]; overexpression of the eps15 tyrosine mutant, however, does not affect EGFR clustering, eps15 recruitment to the plasma membrane and clathrin-coating formation suggesting that tyrosine phosphorylation of eps15 could be a molecular modification required for events following clathrin-coated pit formation, such as vesiculation.

We have recently analyzed the endocytic pathway of the keratinocyte growth factor receptor (KGFR), a receptor tyrosine kinase (RTK) which is a splicing variant of the fibroblast growth factor receptor 2 (FGFR2) [10,11]; we showed that, differently from other members of the FGFR family [12–14], KGFR enters the cell following ligand binding through clathrin-coated pits and that this clathrin-mediated endocytosis leads to rapid internalization of the activated receptors [15,16].

In an attempt to investigate the possible role of eps15 in regulating KGFR uptake at the plasma membrane, we found that eps15, differently from EGFR but similarly to other RTKs such as platelet-derived growth factor receptor and insulin receptor [17], is not tyrosine-phosphorylated by KGFR when activated by its ligand. We decided therefore to compare the pattern of distribution in unstimulated cells and redistribution upon activation of EGFR and KGFR. Our results showed that: (1) eps15 is neither phosphorylated nor recruited to the plasma membrane after KGF-induced KGFR activation and (2) microinjection of an anti-eps15 monoclonal antibody (mAb) blocks EGFR and TfR but not KGFR internalization suggesting that clathrin-dependent ligand-mediated endocytosis, at least in the case of KGFR, can occur without the direct or indirect involvement of eps15.

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2. Materials and methods

2.1. Cell lines

NIH3T3 cells, NIH3T3 EGFR [18] and NIH3T3 KGFR [19] transfected cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum or calf serum plus antibiotics, plated at a density of 8×10^4 cells on round glass coverslips coated with 2% gelatin (Sigma, St. Louis, MO, USA). The human keratinocyte cell line HaCaT was cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics.

For treatment with KGF or with KGF-HFc chimeric protein, obtained by fusion of KGF with the HFc portion of immunoglobulin G [19], cells were serum-starved for 12 h, washed with cold medium, incubated with 100 ng/ml KGF (Upstate Biotechnology, Lake Placid, NY, USA) or with KGF-HFc in DMEM containing 0.3 M NaCl for 1 h at 4°C and immediately fixed or washed with prewarmed medium in the absence of 0.3 M NaCl and incubated at 37°C for an additional 30 min. For EGF treatment, cells were washed with cold medium and incubated with 100 ng/ml EGF (Upstate) or with 2 μg/ml rhodamine-conjugated EGF (Molecular Probes, Eugene, OR, USA) in DMEM at 4°C for 1 h and then warmed to 37°C for an additional 30 min. To induce Tf internalization, cells were incubated with 50 μg/ml Tf-Texas red (Molecular Probes) in DMEM for 10 min at 37°C before fixation.

2.2. Microinjection

Microinjection was performed using an Eppendorf microinjector (Eppendorf, Hamburg, Germany) and an inverted microscope (Zeiss, Oberkochen, Germany). Injection pressure was set at 30–80 hPa and the injection time at 0.3–0.5 s. Anti-eps15 mAb VV1 and control mouse IgG were microinjected into the cytoplasm of the cells at a concentration of 1 mg/ml. At 30 min after injection, cells were incubated with KGF, EGF or Tf as above to induce clathrin-mediated endocytosis.

2.3. Generation and purification of anti-eps15 mAb VV1

An anti-eps15 mouse mAb denominated VV1 was generated using recombinant eps15 glutathione *S*-transferase (GST) fusion protein ([1] and kindly provided by Prof. Di Fiore, Milan, Italy) as antigen source to immunize BALB/c mice. Hybridomas were obtained by fusion of NS-1 myeloma cells with spleen cells from immunized mice. The specificity of hybridomas was analyzed in enzyme-linked immunosorbent assay (ELISA) using pGEX-eps15 and pGEX as negative control (kindly provided by Prof. Di Fiore, Milan, Italy). mAb VV1 (isotype IgG1) was selected on the basis of specific reactivity with the eps15 protein in ELISA. The anti-eps15 mAb VV1 was then further selected in ELISA using a C-terminal eps15 fragment containing the DPF domain and then purified as described [20].

2.4. Immunoprecipitation and Western blot analysis

For the further characterization of the VV1 anti-eps15 mAb, subconfluent cultures of NIH3T3 fibroblasts and confluent cultures of human HaCaT keratinocytes were lysed and subjected to electrophoresis on 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously described [16]. The blots were then incubated for 1 h at room temperature with the anti-eps15 mAb VV1 (at 20 μg/ml) or with the anti-eps15 polyclonal antibodies (pAb) (Santa Cruz) (1/500), followed by enhanced chemiluminescence detection (Amersham, Arlington Heights, IL, USA). For immunoprecipitation, 250 μg of cell lysates was precipitated using 3 μg/ml of mAb VV1, 4 μg of anti-eps15 pAbs (Santa Cruz) or an unrelated murine mAb for 2 h at 4°C, and immune complexes were recovered by absorption to protein A-Sepharose (Amersham). Immunoprecipitated proteins were then processed for immunoblotting with the anti-eps15 full-length pAbs as above. A polyclonal rabbit antiserum directed against the full-length eps15 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as positive control and purified mouse immunoglobulins detecting unrelated antigens and rabbit non-immune serum were used as negative controls.

For the detection of eps15 tyrosine phosphorylation, NIH3T3 KGFR and NIH3T3 EGFR cells, serum-starved for 12 h, were left untreated or treated with 100 ng/ml KGF, 0.3 M NaCl and 100 ng/ml EGF (Upstate Biotechnology) for 10 min at 37°C or for 1 h at 4°C. Following incubation, the cells were lysed as described [16] and 3 mg of total protein was immunoprecipitated with 4 μg of an anti-eps15 pAb (Santa Cruz). The membrane was probed for 1 h with an anti-phosphotyrosine mAb (Upstate Biotechnology) at 1:1000 dilution and then was stripped and reprobed with the anti-eps15 pAbs diluted 1:500.

For the detection of KGFR tyrosine phosphorylation, NIH3T3 KGFR cells, serum-starved for 12 h, were left untreated or treated with KGF as above. 1 mg of total protein was immunoprecipitated with 4 μg of anti-Bek pAbs (C-17; Santa Cruz) as previously described [16]. The membrane was probed for 1 h with anti-phosphotyrosine as above. The filter was stripped and reprobed with the anti-Bek pAbs, diluted 1:500.

For the detection of EGFR tyrosine phosphorylation, NIH3T3 EGFR cells, serum-starved for 12 h, were left untreated or treated with EGF as above. 1 mg of total protein was immunoprecipitated with 4 μg of an anti-EGFR mAb (528; Santa Cruz) and the membrane was probed for 1 h with anti-phosphotyrosine as above. The filter was stripped and reprobed with anti-EGFR pAb, diluted 1:500 (1005; Santa Cruz).

2.5. Immunofluorescence

For conventional immunofluorescence, cells grown on coverslips were fixed in methanol at −20°C for 4 min and, after washing in phosphate-buffered saline (PBS), incubated for 1 h at 25°C with anti-eps15 mAb VV1 (1:100 in PBS). The bound antibodies were vi-

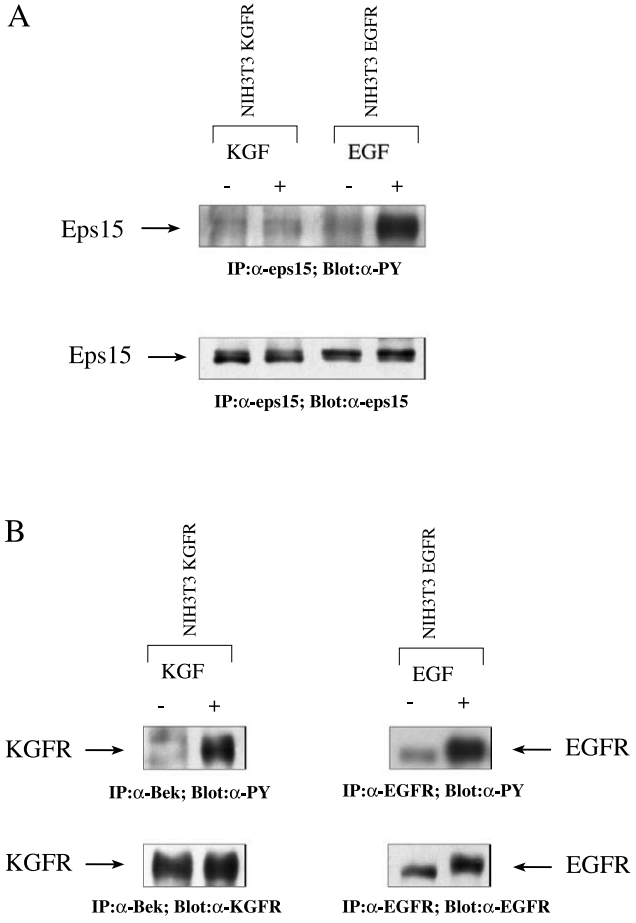


Fig. 1. Tyrosine phosphorylation of eps15 following EGFR but not KGFR activation. NIH3T3 KGFR and NIH3T3 EGFR cells were serum-starved for 12 h and then left untreated or treated with KGF or EGF for 1 h at 4°C. Cell lysates were immunoprecipitated with anti-eps15 antibodies and then immunoblotted with anti-phosphotyrosine (PY) antibody or with anti-eps15 antibodies (A). Alternatively, cell lysates were immunoprecipitated with anti-Bek antibody (B, left) or with anti-EGFR antibody (B, right) and then immunoblotted with anti-PY antibody or with anti-Bek (B, left) or anti-EGFR (B, right) antibodies. Eps15 protein appears tyrosine-phosphorylated in response to EGF treatment, but not in response to KGF, whereas both KGFR and EGFR were tyrosine-phosphorylated in response to the growth factors.

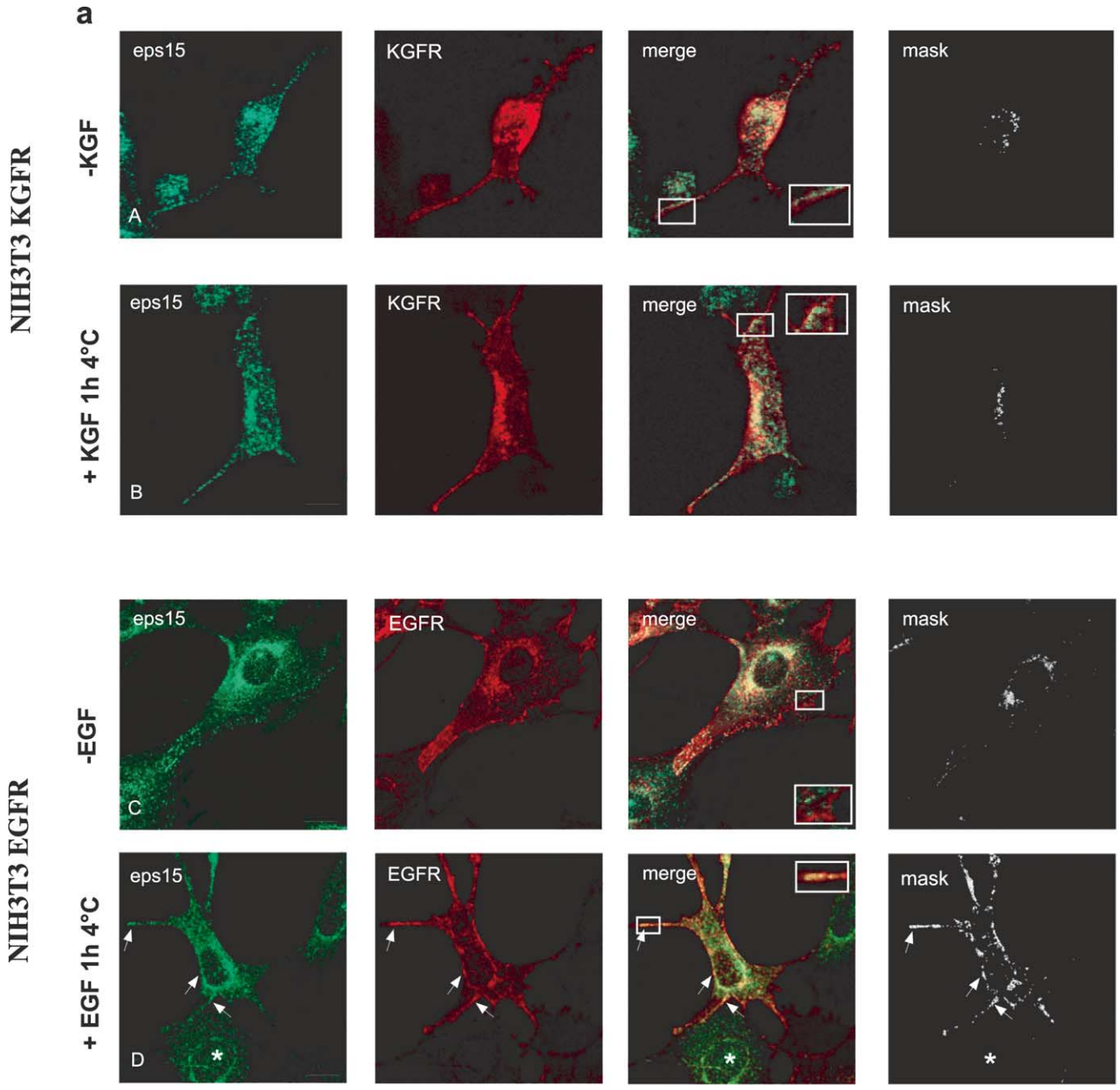


Fig. 2. a: Confocal analysis of the localization of eps15 after KGFR or EGFR activation. NIH3T3 KGFR cells untreated or treated with KGF for 1 h at 4°C were fixed and doubly immunolabeled with anti-eps15 pAbs (green) and anti-Bek mAb (red). Confocal analysis shows that, in untreated NIH3T3 KGFR cells, the eps15 punctate staining does not colocalize with KGFR signal at the cell surface (A) and that the eps15 distribution is not modified by the KGF treatment (B). NIH3T3 EGFR untreated or treated with EGF for 1 h at 4°C was doubly immunolabeled with anti-eps15 pAbs (green) and anti-EGFR mAb (red). In untreated NIH3T3 EGFR, the eps15 staining appears distributed in the cytosol and does not colocalize with the EGFR on the cell plasma membrane (C); after treatment with EGF, the eps15 signal appears partially redistributed and localized under the plasma membrane where colocalization of the protein with EGFR is observed (D, arrows). Eps15 recruitment to the plasma membrane is not evident in cells that express low levels of EGFR (asterisk). The extent of colocalization is shown in yellow after merging or as white dots after masking the images. Bars: 10 μm. b: NIH3T3 KGFR and NIH3T3 EGFR cells treated with KGF-HFc or EGF-TRITC for 1 h at 4°C were doubly immunolabeled with anti-ep15 antibodies. Confocal analysis reveals that eps15 is not recruited to the plasma membrane after KGF-HFc binding and does not colocalize with the KGF-HFc signal distributed on the surface of NIH3T3 KGFR cells (A). In contrast, treatment with EGF-TRITC induces redistribution of eps15 toward the plasma membrane, where the staining corresponding to eps15 colocalizes with the EGF-TRITC signal (B, arrows). Eps15 redistribution to the plasma membrane is not evident in cells that express low levels of EGFR (asterisk). The extent of colocalization is shown in yellow after merging or as white dots after masking the images. Bars: 10 μm.

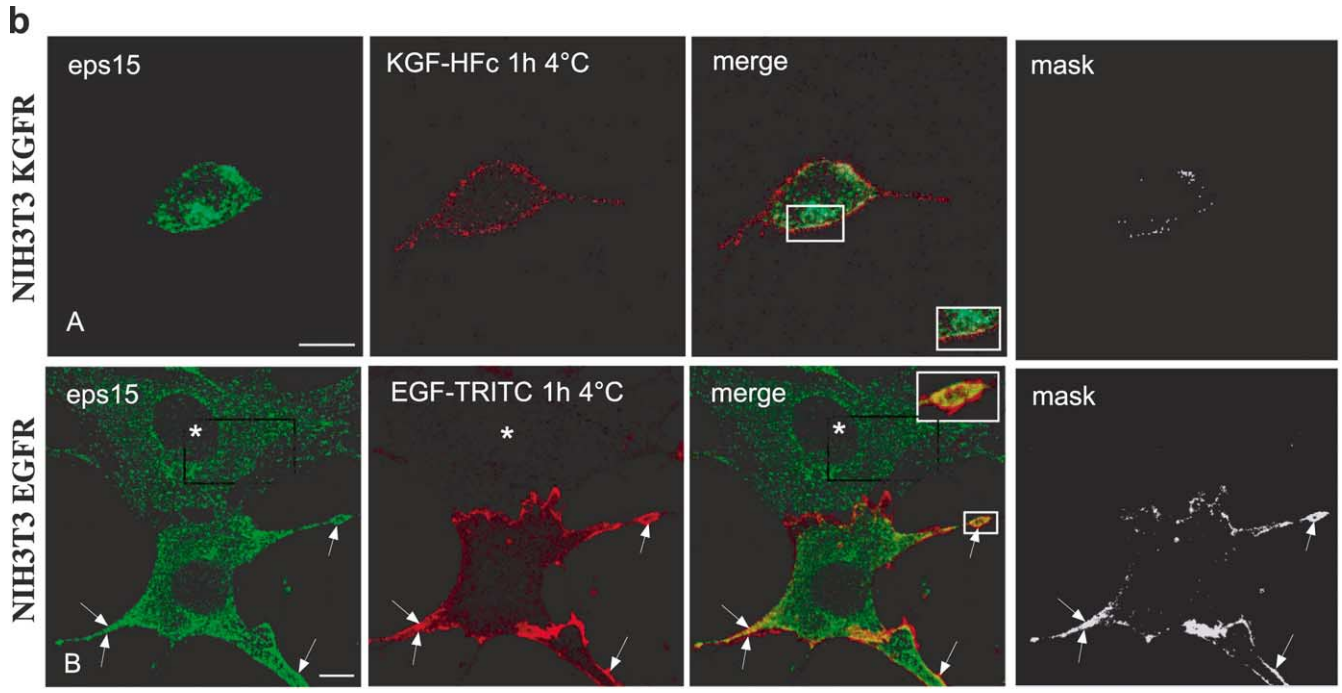


Fig. 2 (*Continued*).

sualized with FITC-conjugated goat anti-mouse IgG (1:50 in PBS) (Cappel Research Products, Durham, NC, USA). In double immunofluorescence experiments after treatment with KGF, KGF-HFc or EGF as above, cells were fixed in methanol at −20°C for 4 min and then incubated with the following primary antibodies: anti-eps15 mAb VV1 (1:100 in PBS), anti-eps15 pAbs (1:100 in PBS) (kindly provided by Prof. P.P. Di Fiore, Milan, Italy), anti-Bek pAbs (1:10 in PBS), anti-Bek mAb (1:10 in PBS) (C8, Santa Cruz) or with anti-EGFR mAb (1:50 in PBS) (528, Santa Cruz). The primary antibodies were visualized using: FITC-conjugated goat anti-mouse IgG as above, FITC-conjugated goat anti-rabbit IgG (1:300 in PBS) (Cappel), Texas red-conjugated goat anti-mouse IgG (1:50 in PBS) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or Texas red-conjugated goat anti-rabbit IgG (1:100 in PBS) (Jackson). KGF-HFc was visualized using FITC-conjugated goat anti-mouse IgG as above. Colocalization of fluorescence signals was evaluated using a Zeiss confocal laser scan microscope. To prevent cross-talk between the two signals, the multitrack function was used.

After microinjection, cells were fixed with 4% paraformaldehyde in PBS (30 min at 25°C) and permeabilized with 0.1% Triton X-100 in PBS for 5 min. To identify injected cells, they were incubated with FITC-conjugated goat anti-mouse IgG as above. Coverslips were finally mounted with 90% glycerol in PBS for observation with a Zeiss Axiophot epifluorescence microscope. Fluorescence signals were analyzed either by recording stained images using a cooled CCD color digital camera SPOT-2 (Diagnostic Instruments, Sterling Heights, MI, USA) and FISH 2000/H1 software (Delta Sistemi, Rome, Italy). Quantitative analysis of the receptor internalization in injected cells was performed evaluating five different areas of each slide randomly taken from three different experiments: results are expressed as percentage of injected cells showing KGFR or EGFR fluorescence signal at the cell plasma membrane.

3. Results

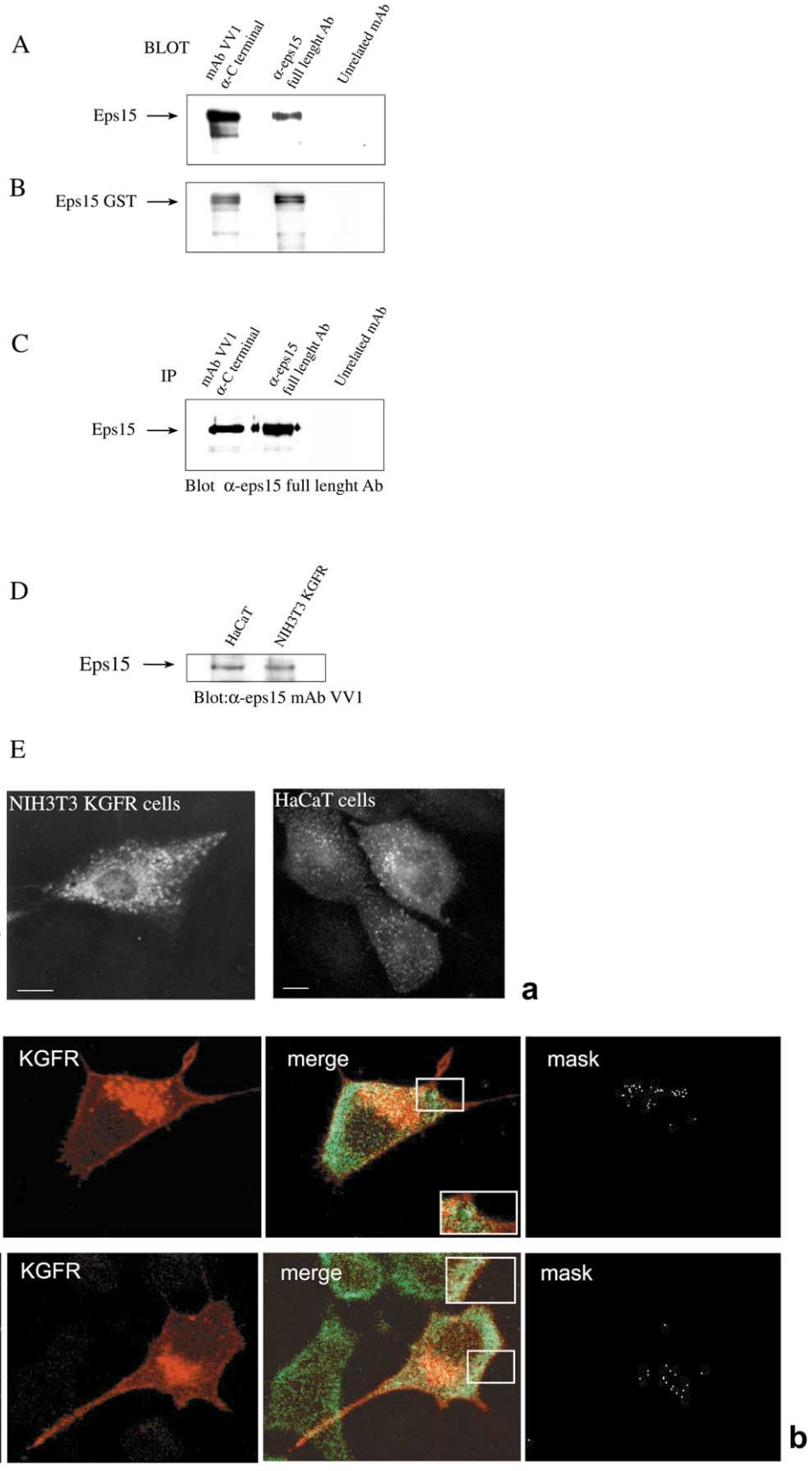
3.1. *Eps15 is not tyrosine-phosphorylated by KGF-induced receptor activation*

To first determine whether eps15 would undergo tyrosine phosphorylation upon KGFR activation at the cell plasma membrane as induced by ligand binding at 4°C, NIH3T3 KGFR cells were serum-starved for 12 h, left untreated or treated with KGF (100 ng/ml) for 1 h at 4°C, then immunoprecipitated with anti-eps15 pAbs and immunoblotted with anti-phosphotyrosine mAb. To compare the possible effect induced by KGFR activation on eps15 phosphorylation with the known effect induced by EGFR activation at 4°C [8], NIH3T3 EGFR cells were serum-starved for 12 h, left untreated or treated with EGF (100 ng/ml) for 1 h at 4°C, and immunoprecipitated with anti-eps15 mAb and then blotted with anti-phosphotyrosine antibody as above. KGF treatment of NIH3T3 KGFR cells resulted in no tyrosine phosphorylation of the eps15 protein (Fig. 1A), although the same treatment was able to induce tyrosine phosphorylation of KGFR as expected [17], assessed by immunoprecipitation with anti-Bek antibodies and Western blot with anti-phosphotyrosine antibodies (Fig. 1B, left panel). In contrast, treatment of NIH3T3 EGFR cells with EGF appeared to trigger tyrosine phosphorylation of both eps15 (Fig. 1A) and EGFR (Fig. 1B, right panel), as previously reported [8]. In order to evaluate if the phosphorylation of eps15 by KGFR could require the recruitment of the receptor into clathrin-coated pits, which is known to be inhibited at 4°C, eps15 phosphorylation was also analyzed after treatment of NIH3T3 KGFR transfectants with KGF for 10 min at 37°C, in comparison with parallel treatment of NIH3T3 EGFR cells with EGF for 10 min at 37°C. However, also in these conditions, eps15 was tyrosine-phosphorylated by EGF, but not by KGF incubation (data not shown).

Thus eps15 does not appear to be tyrosine-phosphorylated by KGF-induced KGFR activation, indicating that eps15 is not a substrate of the KGFR kinase.

3.2. *KGFR activation does not induce recruitment of eps15 to the plasma membrane*

To evaluate if eps15 would be recruited to the cell plasma


A
BLOT
mAb VV1 α-C terminal
α-eps15 full lenght Ab
Unrelated mAb
Eps15
B
Eps15 GST
C
IP
mAb VV1 α-C terminal
α-eps15 full lenght Ab
Unrelated mAb
Eps15
Blot α-eps15 full lenght Ab
D
HaCaT
NIH3T3 KGFR
Eps15
Blot:α-eps15 mAb VV1
E
anti-eps15 mAb VV1
NIH3T3 KGFR cells
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a
-KGF
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eps15
KGFR
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b

membranes following KGFR tyrosine phosphorylation and activation, as previously demonstrated for EGFR [8], we analyzed by immunofluorescence and confocal microscopy the localization of eps15 after KGFR and EGFR activation. NIH3T3 KGFR and NIH3T3 EGFR cells treated or not with KGF and EGF for 1 h at 4°C were fixed and processed for double immunofluorescence with anti-eps15 pAbs and with anti-Bek mAb or anti-EGFR mAb, respectively (Fig. 2a). In untreated cells, eps15 staining appeared in intracellular dots present at the cell periphery as well as in the central areas of the cell cytoplasm, as expected [8] (Fig. 2a, A and C), whereas both KGFR and EGFR signals were distributed mainly on the cell plasma membrane, although intracellular perinuclear staining corresponding to synthesizing receptors was also evident (Fig. 2a, A and C). Confocal analysis showed that the eps15 punctate signal did not colocalize with KGFR (Fig. 2a, A) or EGFR (Fig. 2a, C).

Treatment of NIH3T3 KGFR cells with KGF for 1 h at 4°C did not induce eps15 relocation; in fact, staining for eps15 appeared distributed in dots scattered throughout the entire cytoplasm and did not colocalize with KGFRs present on the cell plasma membrane of the transfected cells (Fig. 2a, B). In contrast, EGF treatment of NIH3T3 EGFR cells at 4°C induced recruitment of eps15 to the plasma membrane (Fig. 2a, D arrows) as demonstrated by colocalization of the signal corresponding to eps15 with EGFRs present on the cell surface (Fig. 2a, D arrows), as expected [8]. Moreover, eps15 recruitment could not be observed in cells expressing only undetectable levels of EGFR among the transfected ones (Fig. 2a, D asterisk).

To confirm our observations and to unequivocally display the recruitment of eps15 at the plasma membrane after EGFR but not after KGFR activation, without the possible interference of intracellular receptors, we used directly or indirectly fluorochrome-conjugated ligands bound at the cell plasma membranes to visualize the activated receptors. NIH3T3 KGFR cells, starved for 12 h, were treated for 1 h at 4°C with a functional KGF-HFc chimeric protein obtained by fusion of KGF with the HFc fragment of IgG which possesses binding and activating properties of the KGF ligand [16,19,21], followed by fixation and incubation with a secondary anti-IgG antibody. In parallel experiments, NIH3T3 EGFR cells were incubated with EGF-TRITC for 1 h at 4°C and immediately fixed. Cells were then processed for double immunofluorescence using anti-eps15 pAbs as above. Confocal analysis showed that eps15 did not colocalize with cell surface KGFRs stained with KGF-HFc (Fig. 2b, A), whereas evident colocalization of EGFRs stained with EGF-TRITC and eps15 was observed (Fig. 2b, B).

Thus KGFR activation, differently from EGFR activation, does not induce either eps15 phosphorylation or protein recruitment to the plasma membrane.

3.3. *KGFR endocytosis is not blocked by microinjection of anti-eps15 mAb*

To directly analyze the possible role of eps15 in regulating the KGFR endocytic process compared to EGFR and to better define the function of the C-terminal DPF domain (containing the AP2 binding L2 region) in this process, we utilized an anti-eps15 murine mAb which has been generated and characterized. First mAb screening was performed by ELISA using the GST eps15 recombinant protein and the related fragments: one murine mAb specifically recognizing the C-terminal DPF domain was selected and named VV1 (data not shown). Fig. 3a illustrates that VV1 mAb is able to detect the eps15 protein either by direct immunoblotting performed on NIH3T3 cell lysates as well as on the GST recombinant protein or by immunoprecipitation of NIH3T3 cell lysates. In fact, Western blot analysis showed that the anti-eps15 mAb VV1 specifically recognizes a protein species corresponding to the molecular mass of the eps15 product (142 kDa) in NIH3T3 cell lysates and the eps15 recombinant protein (Fig. 3a). VV1 mAb was also able to efficiently immunoprecipitate the eps15 protein from the NIH3T3 cell lysates (Fig. 3a). An unrelated mouse purified mAb (anti-CEA) was used as negative control. To verify eps15 expression in normal epithelial cells which are known to express endogenous KGFR, we performed parallel Western blot experiments using the human keratinocyte HaCaT cell line grown to confluence [22]; again the anti-eps15 mAb VV1 specifically recognizes a protein species corresponding to the molecular weight of eps15 in both HaCaT and NIH3T3 cell lysates. In immunofluorescence experiments on both NIH3T3 and HaCaT cells, the anti-eps15 VV1 mAb displayed the typical pattern of staining of the protein characterized by small dots scattered throughout the cell cytoplasm (Fig. 3a), as previously described [8]. Double immunofluorescence and confocal analysis performed in NIH3T3 KGFR transfected cells using the anti-eps15 mAb and an anti-Bek pAb confirmed that eps15 was not recruited to the plasma membrane following treatment with KGF for 1 h at 4°C: the signal corresponding to eps15 did not appear to change its intracellular distribution and to colocalize with KGFRs present at the cell surface (Fig. 3b), as demonstrated above using anti-eps15 pAbs.

To investigate if the anti-eps15 antibody could also block the KGFR endocytic process, we evaluated the effect of mAb VV1 microinjection on NIH3T3 KGFR cells as well as on HaCaT keratinocytes treated, after microinjection, with KGF for 1 h at 4°C followed by warming to 37°C for 30 min to induce KGFR endocytosis. Injected cells were visual-

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Fig. 3. a: Characterization of anti-eps15 mAb VV1. Specific detection of eps15 protein by direct immunoblotting performed on NIH3T3 cell lysates or on GST recombinant eps15 using the mAb VV1, anti-eps15 pAbs or an unrelated mAb (A and B). Immunoprecipitation of NIH3T3 cell lysates with mAb VV1 or anti-eps15 pAbs or an unrelated mAb followed by immunoblot analysis with anti-eps15 pAbs (C). Parallel direct immunoblotting of cell lysates from HaCaT keratinocytes and NIH3T3 fibroblasts with mAb VV1 (D). Immunofluorescence analysis on NIH3T3 cells and on HaCaT keratinocytes performed with anti-eps15 mAb VV1 displays the typical punctate staining of eps15 (E). b: Immunofluorescence and confocal analysis of the localization of eps15 and KGFR after KGF treatment using the anti-eps15 mAb VV1. NIH3T3 KGFR cells untreated (A) or treated with KGF for 1 h at 4°C (B) were doubly immunolabeled with anti-eps15 mAb VV1 and anti-KGFR pAbs. Confocal analysis shows that eps15 is localized in intracellular dots scattered throughout the cytoplasm and does not colocalize with KGFRs in either untreated (A) or KGF-treated cells (B); eps15 distribution is unmodified. The extent of colocalization is shown in yellow after merging or as white dots after masking the images. Bar: 10 μm.

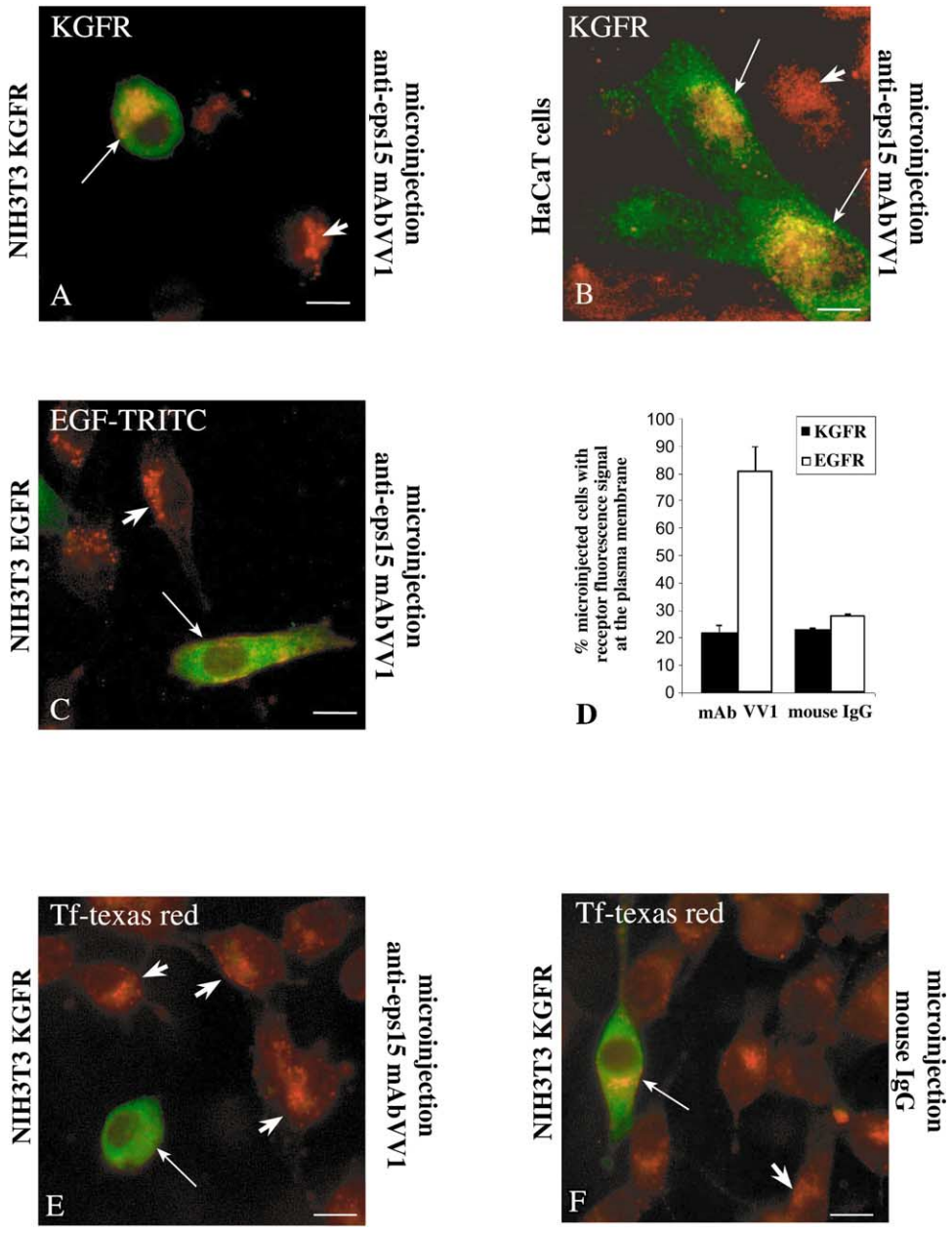

Fig. 4. Effect of microinjected anti-eps15 mAb on KGFR, EGFR and Tf endocytosis. NIH3T3 KGFR cells (A,D,E), HaCaT keratinocytes (B) and NIH3T3 EGFR cells (C) were microinjected with anti-eps15 mAb (A–D) or control mouse IgG (E). After microinjection, cells were incubated with KGF (A,B) or EGF-TRITC (C) for 1 h at 4°C and then warmed for 30 min to 37°C to allow KGFR or EGFR internalization. Alternatively, microinjected NIH3T3 KGFR was treated with Tf-Texas red for 10 min at 37°C in order to permit Tf endocytosis (D,E). At the end of the incubations at 37°C, all microinjected cells were fixed and identified using FITC-conjugated goat anti-mouse IgG. Double immunofluorescence with anti-Bek pAbs shows that the KGFR signal is localized in intracellular perinuclear dots either in cells microinjected with the anti-eps15 mAb (A and B, arrows) or in uninjected cells (A and B, arrowheads). In contrast, EGF-TRITC staining remains distributed on the cell surface in cells microinjected with anti-eps15 mAb (C, arrow), whereas it appears localized in intracellular endocytic dots in uninjected cells (C, arrowhead). Similarly, Tf-Texas red endocytosis appears blocked in cells microinjected with the anti-eps15 mAb (D, arrow), but not in uninjected cells among the injected ones (D, arrowheads) or in cells microinjected with the control mouse IgG (E, arrow). Quantitative analysis of KGFR and EGFR internalization in NIH3T3 transfected cells expressed as percentage of injected cells with receptor fluorescence signal at the cell plasma membrane. Results represent the mean values ± S.D. from three different experiments. Bar: 10 μm.

ized using FITC-conjugated goat anti-mouse IgG. Double immunofluorescence with anti-Bek antibodies showed that, in both microinjected and non-injected cells, the KGFR signal is concentrated in intracellular endocytic structures (Fig. 4A,B). In parallel experiments, NIH3T3 EGFR cells were microinjected with anti-eps15 mAb VV1, treated with EGF-TRITC at 4°C and then warmed for 30 min to 37°C to induce ligand-dependent internalization: the EGF-TRITC signal appeared localized on the cell surfaces of the injected cells, whereas the EGF staining was distributed intracellularly in the uninjected ones (Fig. 4C). Alternatively, NIH3T3 KGFR were microinjected either with anti-eps15 mAb VV1 or with control mouse IgG and incubated with Tf-Texas red for 10 min to 37°C to follow the constitutive internalization of

transferrin. As shown in Fig. 4D, microinjection of the mAb VV1 drastically blocked the Tf-Texas red internalization, whereas Tf endocytosis appeared unaltered in cells microinjected with control mouse IgG (Fig. 4E). Thus, injection of the anti-eps15 mAb is able to interfere with either EGFR or Tf endocytosis but not with KGFR endocytosis.

4. Discussion

Both the endocytic pathways followed by the RTKs of the FGFR family and the mechanisms involved in the regulation of these pathways have been very little investigated and only a few reports have addressed this topic. In addition, some lines of evidence have been presented which indicate that different FGFRs appear to use distinct routes for their internalization [13–15]; among them, only KGFR seems to enter the cells by ligand-induced clathrin-mediated endocytosis [15,16]. In fact, immunoelectron microscopic analysis of KGFR endocytosis has previously revealed that clathrin-coated pits are involved in the ligand–receptor uptake at the plasma membrane [15]; in addition, the selective block of the clathrin-mediated pathway induced by using hypertonic medium and acidification of the cytosol, but not the block of the caveolae-mediated pathway obtained with filipin treatment, appears to be able to inhibit KGFR internalization (Belleudi et al., manuscript in preparation). Because of the similarity of the pathway of KGFR internalization to that followed by EGFR [23], we decided here to investigate the possible role of eps15 in regulating this process. It has been reported that eps15 is recruited to the plasma membrane after EGF-induced EGFR phosphorylation [8] and that microinjection of anti-eps15 antibodies or overexpression of dominant negative domains of the protein blocks EGFR and Tf internalization [6], demonstrating that eps15 plays a general role in both ligand-dependent and constitutive clathrin-mediated endocytosis. It has also been proposed that eps15 tyrosine phosphorylation is not required for the function of the protein in regulating constitutive endocytosis, but it is necessary for the ligand-dependent receptor internalization [9]. Our results, however, demonstrate that: (a) eps15 is not tyrosine-phosphorylated in response to KGF stimulation, despite efficient phosphorylation of the KGFR, and (b) the injection of an anti-eps15 mAb interferes, as expected [6], with the EGFR internalization, but it does not inhibit KGFR internalization, suggesting that the clathrin-mediated process involved in the uptake of KGF–KGFR complexes is not mediated by eps15.

Numerous multivalent proteins and multiple alternative and redundant mechanisms are thought to function in RTK clathrin-mediated endocytosis (for recent reviews see [24,25]). EGFR endocytosis, for example, occurs through clathrin-coated pits even independently of AP2 binding to the activated receptors [26] and adapters and/or signaling substrate proteins other than eps15, such as Grb2 [27], epsin [5] and c-Cbl [28], are possibly responsible for recruitment of AP2 and clathrin to the plasma membrane which triggers coated pit assembly [29,30]. Other substrates could be possibly involved in the regulation of KGFR endocytosis: it has been shown that phospholipase Cγ binds to an autophosphorylation site on FGFR1, a receptor highly related to KGFR, and that the mutation of this tyrosine residue on the receptor causes a block of endocytosis [31]. Moreover, it has been shown that the other major substrate of FGFR, the FRS2α protein, promotes the ubiquitination of FGFR1 by recruitment of Cbl and controls, at least in part, the down-regulation of the receptor [32]. Therefore, it is not surprising that eps15 does not appear to be involved either directly or indirectly in KGFR internalization, although further work is needed to elucidate the molecular mechanisms of this process of receptor uptake. However, our results indicate that not only eps15 phosphorylation but also its recruitment to the plasma membrane and presumably its targeting to clathrin-coated pits are not always required for ligand-induced endocytosis and that KGFR may represent a model system for the study of alternative pathways of clathrin-dependent regulated endocytosis which may occur independently of eps15 and possibly of eps15-interacting proteins.

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