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Research Article

Hrs regulates the endocytic sorting of the fibroblast growth factor receptor 2b

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ABSTRACT

The keratinocyte growth factor receptor or fibroblast growth factor receptor 2b (KGFR/FGFR2b) is activated by the specific interaction with the keratinocyte growth factor (KGF/FGF7), which targets the receptor to the degradative pathway, and the fibroblast growth factor 10 (FGF10/KGF2), which drives the receptor to the juxtanuclear recycling route. Hrs plays a key role in the regulation of the endocytic degradative transport of ubiquitinated receptor tyrosine kinases, but the direct involvement of this protein in the regulation of FGFR endocytosis has not been investigated yet. We investigated here the possible role of Hrs in the alternative endocytic pathways of KGFR. Quantitative immunofluorescence microscopy and biochemical analysis showed that both overexpression and siRNA interference of Hrs inhibit the KGF-triggered KGFR degradation, blocking receptor transport to lysosomes and causing its rapid reapparance at the plasma membrane. In contrast, the FGF10-induced KGFR targeting to the recycling compartment is not affected by Hrs overexpression or depletion. Coimmunoprecipitation approaches indicated that Hrs is recruited to KGFR only after KGF treatment, although it is not tyrosine phosphorylated by the ligand. In conclusion, Hrs regulates the KGFR degradative pathway, but not its juxtanuclear recycling transport. In addition, the results suggest that Hrs recruitment to the receptor, but not its ligand-induced phosphorylation, could be required for its function.

Introduction

The plasma membrane signaling of receptor tyrosine kinases (RTKs) is attenuated by receptor endocytosis and degradation and the targeting of internalized receptors to the degradative pathway requires their ubiquitination [1]. In the early/sorting endosomes ubiquitinated receptors are sorted to internal vesicles of multivesicular bodies (MVBs) [2] and are then transported to lysosomes for degradation.

The fibroblast growth factor receptors (FGFRs) appear to follow multiple endocytic pathways and the receptor ubiquitination level has been proposed as the main molecular event able to regulate their sorting. In fact, the FGFR1, 2 and 3 are consistently ubiquitinated and sorted to lysosomes for degradation, while FGFR4, which is poorly ubiquitinated, is efficiently transported from sorting endosomes to the recycling compartment [3] an endocytic organelle that is typically located in close proximity to the nucleus and the microtubule organizing centre [4,5].

Our previous studies on the endocytic pathways followed by KGFR, a splicing transcript variant of FGFR2 expressed exclusively on epithelial cells [6], have demonstrated that: i) KGFR is internalized by clathrin-coated pits after treatment with the two ligands KGF/FGF7 and FGF10/KGF2 [7,8]; ii) after internalization, KGF targets the receptor to the degradative pathway, whereas

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FGF10 induces KGFR sorting to the recycling route [9]; iii) the alternative fate of KGFR is dependent on a different ligand-induced receptor ubiquitination mediated by the ubiquitin ligase c-Cbl [9].

The hepatocyte growth factor-related tyrosine kinase substrate (Hrs) is a key endocytic regulator which recognizes the ubiquitinated receptors through its UIM domain [10,11] and mediates receptor sorting onto internal vesicles of multivesicular bodies (MVBs) [2,12], acting upstream ESCRT complexes [2,13,14]. Several reports have shown that Hrs plays a crucial role in the regulation of RTK degradative sorting: in fact, Hrs depletion or overexpression causes receptor accumulation in clusterized early endosomes and in MVBs [12,13,15,16], blocking receptor transport to lysosomes and enhancing direct recycling of ubiquitinated receptors to the plasma membrane [17]. However, it has been recently demonstrated that Hrs is also involved in the control of endocytic pathways that are not mediated by ubiquitination, such as the degradative and the recycling transport of G protein-coupled receptors [18,19].

Since KGFR/FGFR2b is able to follow different endocytic trafficking in response to its ligands, we focused on this receptor as a model to analyze the possible involvement of Hrs in the regulation of FGFR endocytosis and to investigate whether Hrs might play a role not only in the degradative pathway of FGFRs, but also in their ubiquitin-independent sorting through the recycling compartment.

Materials and methods

Cell lines

NIH3T3 EGFR and NIH3T3 KGFR transfected cells were cultured in Dulbecco's DMEM, supplemented with 10% FCS plus antibiotics. NIH3T3 and HeLa cells were transiently transfected or cotransfected with pCI-neo vector containing human KGFR cDNA and pcDNA3 vector containing the constructs FLAG-Hrs and GFP-Hrs (kindly provided respectively by Dr. Cesareni, Rome, Italy and Dr. Urbè, Liverpool, UK) using polifect transfection reagent (Qiagen, Valencia, CA, USA). Transfected cells were collected 48 h after transfection for evaluation of protein expression and internalization assays. For treatment with growth factors, cells were serum starved for 12 h and then incubated with 100 ng/ml KGF (Upstate, Lake Placid, NY, USA), 100 ng/ml FGF10 (PeproTech, London, UK) + 0.3 μg/ml heparin, 100 ng/ml PDGF (PeproTech) or with 100 ng/ml EGF (Upstate) for different times at 37 °C to induce receptor activation. Alternatively, cells were starved for 12 h, washed with cold medium, incubated with 100 ng/ml KGF or with 100 ng/ml FGF10 + 0.3 μg/ml heparin for 1 h at 4 °C and immediately fixed or washed with prewarmed medium and incubated at 37 °C for different times to induce KGFR endocytosis and recycling before fixation. To induce transferrin (Tf) internalization, cells were incubated with 50 μg/ml Transferrin-Texas Red (Tf-TxRed) (Molecular Probes, Eugene, OR, USA) at 37 °C for 19′ or 1 h before fixation. To induce LysoTracker internalization, cells were incubated with 100 nM LysoTracker-Red (Molecular Probes) for 1 h at 37 °C. To inhibit the synthesis of proteins, cells were treated with 25 μg/ml cycloheximide (Sigma Chemicals, St. Louis, Mo., USA) for 4 h at 37 °C. To visualize cell surface, plasma membranes were decorated with FITC-conjugated lectin wheat germ agglutinin (WGA) (Sigma) at 4 °C before fixation and permeabilization.

For RNA interference and Hrs silencing, HeLa cells were transfected with 125 pM of small interfering RNA (siRNA) for Hrs (Santa Cruz Biotechnology Inc., Santa Cruz, CA) using lipofectamine 2000 according to the manufacturer's protocol. 24 h after transfection, siRNA transfected and untransfected cells were processed for Western blot analysis.

Microinjection

Microinjection was performed with an Eppendorf microinjector (Eppendorf, Hamburg, Germany) and an inverted microscope (Zeiss, Oberkochen, Germany). Injection pressure was set at 80–

Fig. 1 – Hrs recruitment to KGFR. (A) NIH3T3 and HeLa cells were lysed and processed for Western blot analysis using anti-Hrs polyclonal antibodies to detect the endogenous protein (top): a band of 105 kDa corresponding to the molecular weight of Hrs is visible in both cells. NIH3T3 KGFR cells were serum starved, treated with the KGF or FGF10 for 8′ at 37 °C, lysed and immunoprecipitated with anti-Hrs polyclonal antibodies or with rabbit normal IgG as negative control: immunoblot with anti-phosphotyrosine (PY) monoclonal antibody shows that neither KGF nor FGF10 are able to induce Hrs tyrosine phosphorylation (right-bottom). To assess equal loading, blots were stripped and reprobed with anti-Hrs antibodies. NIH3T3 KGFR and NIH3T3 EGFR cells were serum starved, treated with the indicated growth factors as above, lysed and immunoprecipitated with anti-phosphotyrosine (PY) monoclonal antibody (left-bottom): immunoblot with anti-Hrs polyclonal antibodies shows that PDGF but not KGF or FGF10 treatment increases the slow basal level of Hrs tyrosine phosphorylation visible in untreated NIH3T3 KGFR cells. EGF treatment increases Hrs phosphorylation in NIH3T3 EGFR cells. (B) NIH3T3 KGFR and NIH3T3 KGFR/FLAG-Hrs cells were lysed and processed for Western blot analysis using anti-FLAG polyclonal antibodies and anti-Bek polyclonal antibodies to determine transfection efficiency (top): the band corresponding to exogenous FLAG-Hrs is visible in NIH3T3 KGFR/FLAG-Hrs but not in NIH3T3 KGFR cells, while the band of 140 kDa corresponding to the molecular weight of KGFR is evident in both cells. NIH3T3 KGFR/FLAG-Hrs cells were serum starved, treated with KGF or FGF10 as above, lysed and immunoprecipitated with anti-PY monoclonal antibody, followed by immunoblot with anti-FLAG polyclonal antibodies. Alternatively, cells were immunoprecipitated with anti-Hrs polyclonal antibodies followed by immunoblot with anti-PY antibody: no significant increase in tyrosine phosphorylation of the overexpressed Hrs is detectable after either KGF or FGF10 treatment, although either growth factors efficiently stimulate receptor phosphorylation (right panel). Immunoprecipitation with anti-Bek monoclonal antibody, followed by immunoblot with anti-FLAG or with anti-Hrs antibodies in both NIH3T3 KGFR/FLAG-Hrs cells and HeLa KGFR/FLAG-Hrs reveals coimmunoprecipitation of Hrs with KGFR after KGF but not FGF10 treatment (bottom). The intensity of the bands was evaluated by densitometric analysis and the values from a representative experiment out of three were normalized and expressed as fold increase with respect to the control value. To assess equal loading, blots were stripped and reprobed with anti-Bek antibodies.