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Disruption of the annexin A1/S100A11 complex increases the migration and clonogenic growth by dysregulating epithelial growth factor (EGF) signaling ☆

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ABSTRACT

Endocytosis of activated growth factor receptors regulates spatio-temporal cellular signaling. In the case of the EGF receptor, sorting into multivesicular bodies (MVBs) controls signal termination and subsequently leads to receptor degradation in lysosomes. Annexin A1, a Ca^{2+}-regulated membrane binding protein often deregulated in human cancers, interacts with the EGF receptor and is phosphorylated by internalized EGF receptor on endosomes. Most relevant for EGF receptor signal termination, annexin A1 is required for the formation of internal vesicles in MVBs that sequester ligand-bound EGF receptor away from the limiting membrane. To elucidate the mechanism underlying annexin A1-dependent EGF receptor trafficking we employed an N-terminally truncated annexin A1 mutant that lacks the EGF receptor phosphorylation site and the site for interaction with its protein ligand S100A11. Overexpression of this dominant-negative mutant induces a delay in EGF-induced EGF receptor transport to the LAMP1-positive late endosomal/lysosomal compartment and impairs ligand-induced EGF receptor degradation. Consistent with these findings, EGF-stimulated EGF receptor and MAP kinase pathway signaling is prolonged. Importantly, depletion of S100A11 also results in a delayed EGF receptor transport and prolonged MAP kinase signaling comparable to the trafficking defect observed in cells expressing the N-terminally truncated annexin A1 mutant. These results strongly suggest that the function of annexin A1 as a regulator of EGF receptor trafficking, degradation and signaling is critically mediated through an N-terminal interaction with S100A11 in the endosomal compartment. This interaction appears to be essential for lysosomal targeting of the EGF receptor, possibly by providing a physical scaffold supporting inward vesiculation in MVBs. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

1. Introduction

Growth factor receptor activation triggers a complex network of signaling events that requires accurate tempo-spatial regulation. To avoid constitutive signaling, endocytosis and lysosomal targeting terminates cellular signaling of activated cell surface receptors. In many cases ligand-induced activation of the surface receptor induces endocytosis. Ligand-bound receptors are transported to early endosomes where they are sorted either for recycling to the plasma membrane or for degradation in the lysosomal compartment [1,2]. An important mechanism during the transport along the endocytic pathway is the sorting of activated signaling receptors into multivesicular endosomes [3]. Through budding of vesicles into the endosomal lumen, receptors are sequestered into these intraluminal vesicles and thereby efficiently sorted away from molecules destined for recycling. In the case of activated epidermal growth factor (EGF) receptor (EGFR) this sorting into multivesicular endosomes is essential for signal termination [4]. Several cellular components including the endocytic adaptors Eps15, Hrs and the ESCRT machinery of proteins have been described to participate in the sorting of activated and internalized EGFR. Furthermore, several members of the annexin protein family, including annexins (Anx) A1, A2, A6 and, as we showed recently, AnxA8 have been implicated in EGFR signal termination and lysosomal degradation [5–8]. This involves a specific role for AnxA1, which affects EGF-stimulated inward vesiculation of EGFR containing membrane domains during the formation of intraluminal vesicles in multivesicular bodies (MVBs) [9]. However, although internalization of activated receptors into intraluminal vesicles is a critical mechanism linking degradation and signal termination, the complex molecular mechanisms underlying this process remain to be fully characterized.

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The EGFR belongs to the ErbB family of receptor tyrosine kinases and is a central player in cellular signaling events that control a wide range of processes [10]. Identification of activated EGFR on endosomes that is associated with various downstream effectors led to the concept of "signaling endosomes" [11]. Recent studies not only suggest that endosomal trafficking is important for exploiting the full biological activity of EGFR, but also reveal that EGFR-mediated signaling controls the endocytic pathway itself [12]. Targeting EGFR to internal vesicles of nascent multivesicular endosomes is mediated by ubiquitination of the cytoplasmic receptor domain [13]. Recognition of the ubiquitinated receptor by the Endosomal Sorting Complex Required for Transport (ESCRT) component Hrs (Hepatocyte growth factor Regulated tyrosine kinase Substrate) ultimately leads to receptor concentration into clathrin-coated regions [14]. While sequential interactions of ESCRT-I, -II and -III with ubiquitinated receptors have been intensively studied, the mechanisms concentrating receptors at sites of vesicle formation and mediating vesicle budding at these sites are not yet fully understood [4]. Annexin A1 has been implicated in linking these concentrated receptors to the inward vesiculation process [15]. It is a member of the annexin family of Ca^{2+}-regulated membrane binding proteins that participate in different membrane-related events including membrane organization and vesicular trafficking, some of those related to the regulation of EGFR signaling and trafficking [5–8,16]. AnxA1 expression is deregulated in various cancers and in this context has been linked to signal transduction, tumor invasion and cellular differentiation and proliferation [17,18]. Interestingly, AnxA1 is down-regulated in breast cancer [19], whereas up-regulation of the EGFR is often associated with breast cancer progression [20].

Structurally, AnxA1 is composed of the highly conserved annexin core domain that mediates Ca^{2+}-dependent phospholipid binding and a unique N-terminal domain that modulates the membrane binding specificity of the core domain [21] and harbors sites for posttranslational modification, most notably a tyrosine at position 21 that is phosphorylated by internalized EGFR [22,23]. In membrane-bound AnxA1 the N-terminal domain faces the cytoplasm and is available for specific protein–protein interactions. The best characterized ligand of this N-terminal domain is the EF-hand-type Ca^{2+}-binding protein S100A11 which itself forms a dimer and can thereby bridge two membrane-bound AnxA1 moieties [24–26]. Furthermore, the AnxA1 binding partner S100A11 has recently been demonstrated to represent a novel diagnostic marker for breast carcinoma [27]. Together with the reported role of AnxA1 in mediating the EGF-stimulated inward vesiculation in MVBs [9] these expression studies suggest a function of the protein and possibly the AnxA1/S100A11 complex in tumorigenesis of breast cancer [28].

Despite association of S100A11 with AnxA1 on early endosomes [29], it is not known if AnxA1/S100A11 interaction is involved in the regulation of EGFR trafficking along the endocytic pathway. In this study we demonstrate that binding of S100A11 to the N-terminal domain of AnxA1 is crucially involved in regulating lysosomal degradation of the EGFR. Both depletion of S100A11 and overexpression of an N-terminal AnxA1 deletion mutant lacking the S100A11 binding site resulted in delayed transport of internalized EGF along the endocytic pathway which impaired lysosomal degradation of the EGFR. Thus, modulation of AnxA1 by EGFR phosphorylation and S100A11 binding are important events in regulating the lysosomal degradation of the EGFR.

2. Materials and methods

2.1. Cell culture, transfection and antibodies

HeLa and A431 cells (both expressing endogenous AnxA1, data not shown) were maintained in Dulbecco's modified Eagle's medium High Glucose (PAA) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 7% CO_2 and 37 °C. Cells grown on coverslips were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and further cultivated for 24 hours. Expression constructs of AnxA1full-length–GFP and AnxA1core–GFP have been described previously [21]. For siRNA-mediated S100A11 gene silencing, the S100A11-specific SMARTpool reagent (Dharmacon) consisting of four S100A11-targeting siRNAs was used. AllStars negative control siRNA (Qiagen) was used as a non-silencing control. For transfection of HeLa cells with siRNA we employed oligofectamine (Invitrogen) and cultivated the transfected cells for 72 hours.

Mouse monoclonal antibodies against human lysosome-associated membrane protein 1 (LAMP1) were obtained from the Developmental Studies Hybridoma Bank (clone H4A3; University of Iowa, Iowa City, Iowa, USA). Rabbit polyclonal anti-human S100A11 antibodies were obtained from Proteintech Europe, rabbit polyclonal anti-EGFR antibodies and rabbit polyclonal anti-Rab5 antibodies from Santa Cruz Biotechnology, mouse monoclonal anti-β-actin antibody from Sigma, and mouse monoclonal anti-GFP antibody from Invitrogen. Mouse monoclonal anti-phospho-tyrosine (P-Tyr-100) antibodies, mouse monoclonal anti-phospho-MEK1/2 antibodies, rabbit polyclonal anti-MEK1/2 antibodies, mouse monoclonal anti-phosphoERK1/2 antibodies and rabbit polyclonal anti-ERK1/2 antibodies were purchased from Cell Signaling Technology.

2.2. EGF and transferring uptake

Serum-starved cells were incubated with either 2 μg/ml Texas Red-coupled EGF (Invitrogen) for 10 min or with 50 μg/ml Texas-Red conjugated transferrin (Invitrogen) for 5 or 30 min in serum-free culture medium. After washing with serum-free medium cells were either fixed immediately or further cultivated for 15 or 30 min. All incubation steps were conducted at 37 °C.

2.3. Immunocytochemistry and confocal fluorescence microscopy

HeLa cells grown on coverslips were fixed with 4% paraformaldehyde (PFA) containing 0.2% Triton X-100 for 3 min and subsequent incubation with 4% PFA for 10 min. Cells were blocked with 2% bovine serum albumin (BSA) in PBS followed by incubation with the primary antibody for 45 min at room temperature. After intensive washing with PBS, cells were incubated with the respective secondary antibody conjugated with Cy5. An LSM 510 META microscope (Carl Zeiss) with a Plan-Apochromat 63×/1.4 oil immersion objective was used for confocal microscopy.

2.4. Colocalization of EGF with LAMP1

For colocalization analysis images of a confocal plane in the lower part of 20 transfected cells were acquired for each condition. The amount of colocalization was calculated using BioImageXD [30] and statistical significance of the results from four independent experiments was evaluated by paired Student's *t* test. $P \leq 0.05$ indicated a significant difference.

2.5. Analysis of EGFR degradation and activation

Serum-starved HeLa and A431 cells were incubated with EGF (A431: 10 ng/ml, HeLa: 100 ng/ml, Biomol Research Laboratories) and 10 μg/ml cycloheximide for 15, 30 or 60 min. After stimulation, cells were scraped in ice-cold PBS and pelleted. Cell pellets were resuspended in 8 M urea and sonicated. Equal protein amounts were analyzed by SDS-PAGE followed by immunoblotting with actin serving as an internal loading control. Quantification of signal intensities was carried out by densitometric analysis using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Statistical significance of results obtained from at least three independent experiments was evaluated using the