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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

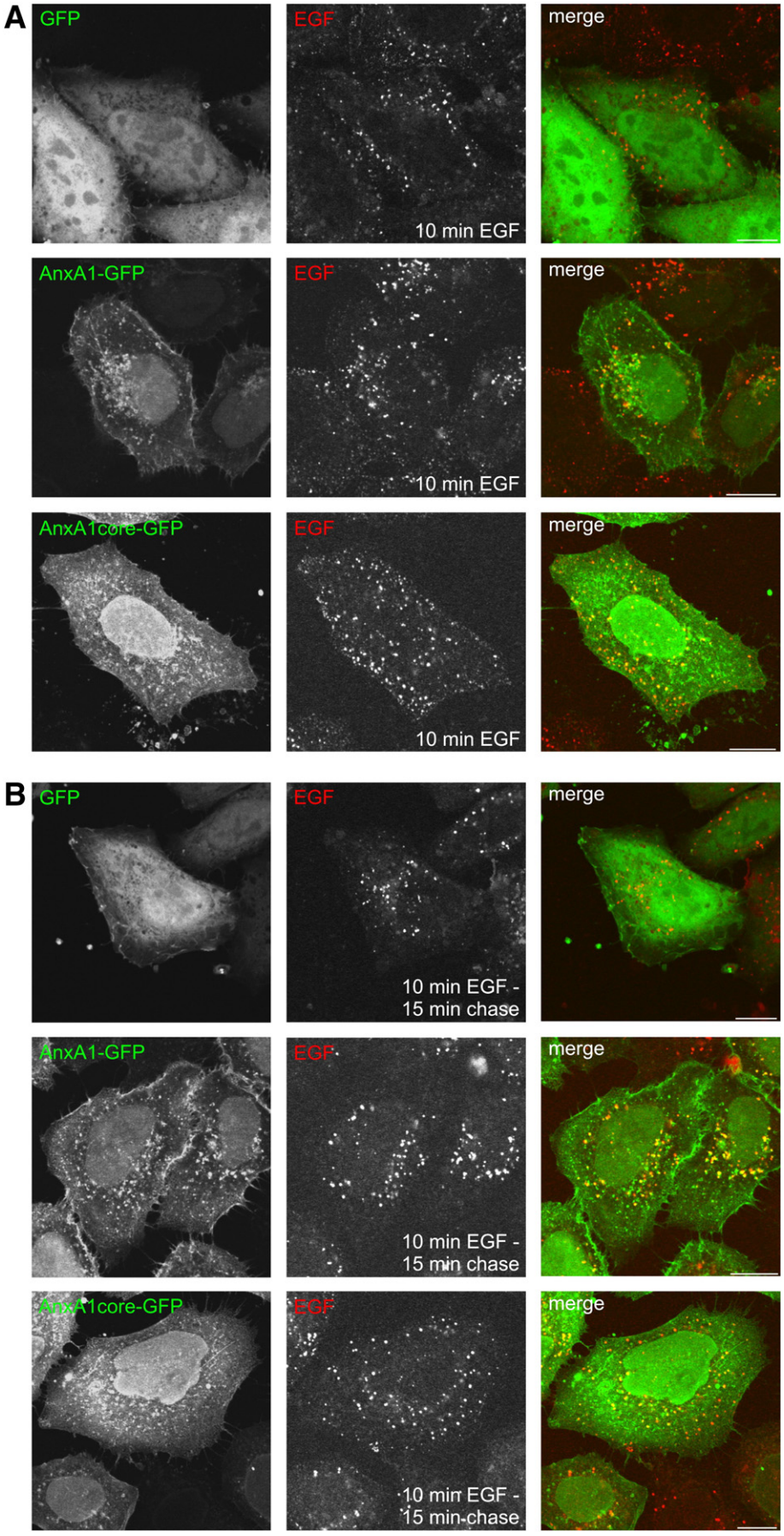
HeLa cells grown on coverslips were fixed with 4% paraformaldehyde (PFA) containing 0.2% Triton X-100 for 3 min and subsequent incubation with 4% PFA for 10 min. Cells were blocked with 2% bovine serum albumin (BSA) in PBS followed by incubation with the primary antibody for 45 min at room temperature. 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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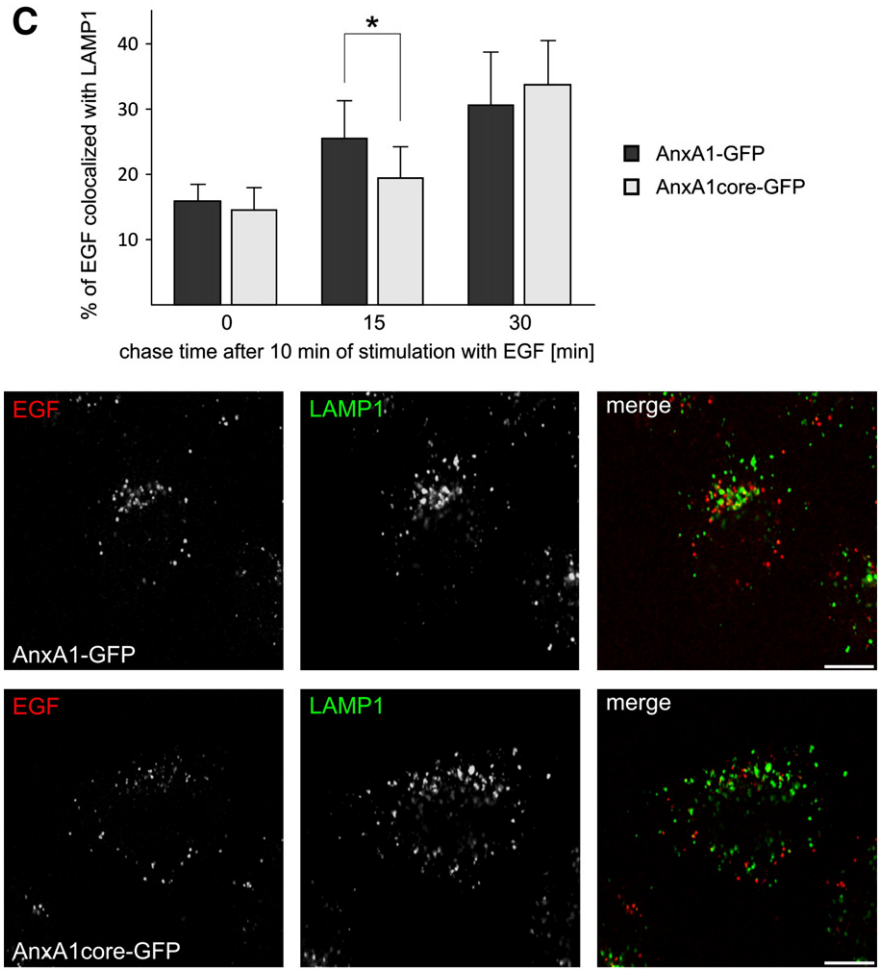


Fig. 1 (*continued*).

Student's *t* test. $P \leq 0.05$ indicated a significant difference. To investigate activation of EGFR and the MAP kinase pathway, cell lysates were separated by SDS PAGE and immunoblots were analyzed using the appropriate antibodies for activated EGFR, MEK1/2 and ERK1/2, respectively.

2.6. Subcellular fractionation by sucrose step gradient

Fractions of early and late endosomes were obtained by flotation in a sucrose step gradient as described previously [31] and protein concentrations in the fractions were measured with the Bradford assay. Equal amounts of protein were immunoblotted for the presence of AnxA1–GFP, AnxA1core–GFP and Rab5 using the appropriate antibodies. Rab5 served as a marker for the quality of early endosomal enrichment. For each gradient, the ratios of the late endosomal/early endosomal GFP signal intensities were calculated. Data were collected from three independent transfections and the statistical significance was evaluated by paired Student's *t* test.

2.7. AnxA1/S100A11 interaction using surface plasmon resonance (SPR)

Recombinant human S100A11 expressed and purified as described previously [25] was dialysed against HBS-P for SPR interaction analysis and protein concentration was determined using the Bradford assay.

SPR experiments were carried out on a Biacore 3000 instrument (GE Healthcare). HBS-P (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% v/v Tween 20) filtered through 0.22 μM filters (Millipore) and degassed was used as running buffer. Purified bovine AnxA1 (BioDesign Int) was immobilized via the amine coupling method on a carboxy-methyl dextran CM5 chip at a flow rate of 5 μl/min following the manufacturer's instructions. Briefly, a blank surface of a CM5 chip was activated with a 7 min injection of a 1:1 mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.05 M N-hydroxysuccinimide (NHS) and approximately 900 response units (RU) of AnxA1 in 10 mM acetate buffer, pH 5.0, were immobilized by injection of protein solution (30 μg/ml). The surface was deactivated via a subsequent 7 min injection of 1.0 M ethanolamine, pH 8.5, to

Fig. 1. Transport of EGF to the late endosomal compartment is retarded in HeLa cells expressing the AnxA1 core domain. (A) Cells expressing GFP, full-length AnxA1 or the N-terminally truncated mutant AnxA1core–GFP were serum-starved and allowed to internalize Texas Red-coupled EGF for 10 min. (B) Upon an additional 15 min chase period, EGF positive endosomes were more dispersed in cells expressing AnxA1core–GFP. (C) The colocalization of the late endosomal/lysosomal marker LAMP1 with EGF was quantified from confocal images of cells expressing either full-length or truncated AnxA1–GFP that had been subjected to a 10 min EGF pulse, followed by 15 or 30 min of chase. For each expression construct and time point, 20 cells were evaluated and the results are expressed as the mean percentages ± SEM from four independent experiments. Paired Student's *t*-test was employed for analysis of statistical significance ($^*P \leq 0.05$). Lower panel: representative confocal sections of cells after a chase period of 15 min are shown. At this time point, significantly less colocalization of EGF with the late endosomal/lysosomal marker LAMP1 was observed in AnxA1core–GFP expressing cells. Note the more peripheral distribution of the endosomes. For a better visualization of the colocalization, the original LAMP1 signals are converted to green. All images shown represent single confocal sections. Bars, 10 μm.

block free ester groups. As reference a flow cell was activated via EDC/NHS and deactivated with ethanolamine but no protein was immobilized. During measurements the response in this flow cell was subtracted from responses in all other flow cells. For interaction analysis, different concentrations of purified S100A11 diluted in HBS-P containing 1 mM Ca^{2+} were injected for 3 min following a dissociation time of 6 min at a flow rate of 40 μl/min. HBS-P containing 1 mM Ca^{2+} was used as running buffer and sensorgrams (response units versus time) were recorded at 25 °C. A 10 μl pulse injection of 10 mM EDTA was used to wash away dissociated S100A11 and to regenerate the surface. Experiments were repeated three times with double injections. The response at equilibrium resonance (R_{eq}) of a series of S100A11 injections was plotted against its concentrations (M) and the K_D value was calculated via the BIAEvaluation Software 4.1 (Biacore AB).

2.8. Migration assay

For cell migration assays, 1×10^6 HeLa cells transiently expressing AnxA1–GFP or AnxA1core–GFP were seeded into cylinders placed in a 48-well plate. Cells were allowed to attach for 2 hours at 7% CO_2 and 37 °C in serum-depleted medium with or without the addition of 100 ng/ml EGF. After removal of the cylinders the cells were rinsed and the respective medium was added. Three wells were used for each condition. The area covered by the cells was recorded directly after the

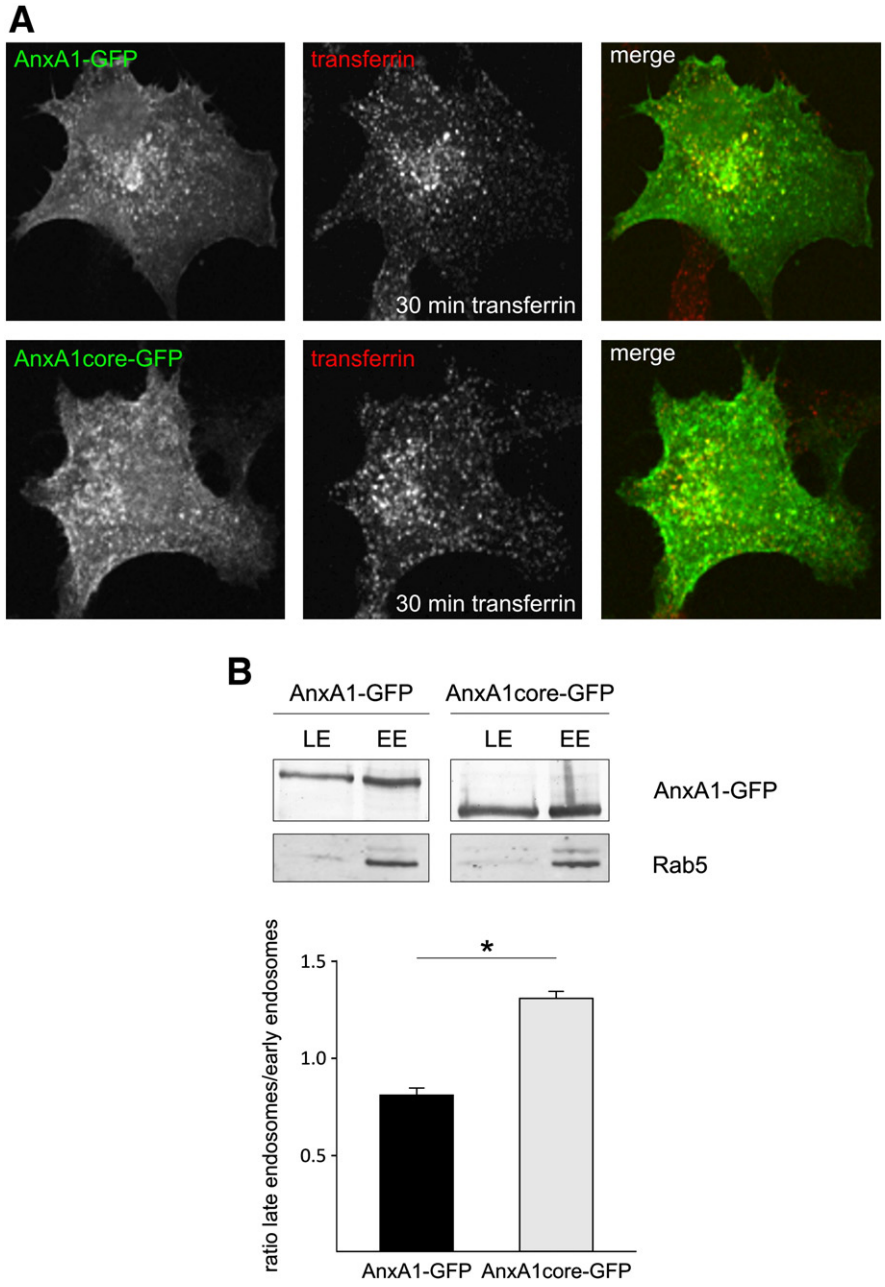


Fig. 2. AnxA1 distribution on endosomes. (A) HeLa cells expressing AnxA1–GFP or AnxA1core–GFP were allowed to internalize Texas Red-coupled transferrin for 30 min to specifically label both the early and recycling endosomes. Note that the distribution of these transferrin receptor enriched compartments appears similar. (B) Endosomes of cells transfected with AnxA1–GFP or AnxA1core–GFP were separated by flotation on a sucrose step gradient and analyzed by immunoblotting for the presence of the respective fusion protein in the late (LE) or early (EE) endosomal fraction. The early endosomal marker Rab5 served as a control for efficient endosome enrichment. GFP signals in the fractions were densitometrically quantified and the enrichment on early endosomes was calculated as ratio LE/EE. Mean values ± SEM from three independent experiments are shown. * indicates a statistically significant difference ($P \leq 0.05$) as revealed by paired t test.

removal of the cylinder and 24 hours later. The cell-covered areas (% of the total area) from four independent experiments were quantified by ImageJ software. The statistical significance was evaluated by paired Student's *t*-test with $P \leq 0.05$ indicating a significant difference.

2.9. Clonogenic assay

A431 cells expressing either AnxA1–GFP or AnxA1core–GFP were seeded in 6-well plates (5×10^3 cells/well) and grown in DMEM, 1% FCS with or without 10 ng/ml EGF for 1–2 weeks. Cells were then fixed and stained with DIFF-QUIK Staining Set (Siemens). Colonies containing more than 40 cells were quantified using ImageJ software. Data were collected from four independent experiments and the statistical significance was evaluated by repeated measures one-way ANOVA followed by Tukey's multiple comparison test. $P \leq 0.05$ indicated a significant difference.

3. Results

3.1. N-terminally truncated AnxA1 delays EGF transport and lysosomal degradation

The annexin A1 N-terminal domain contains two important sites for posttranslational modification, tyrosine 21 that is phosphorylated by internalized EGFR [22,23], and the S100A11 binding site consisting of residues 1–12. Phosphorylation at tyrosine 21 is discussed to trigger

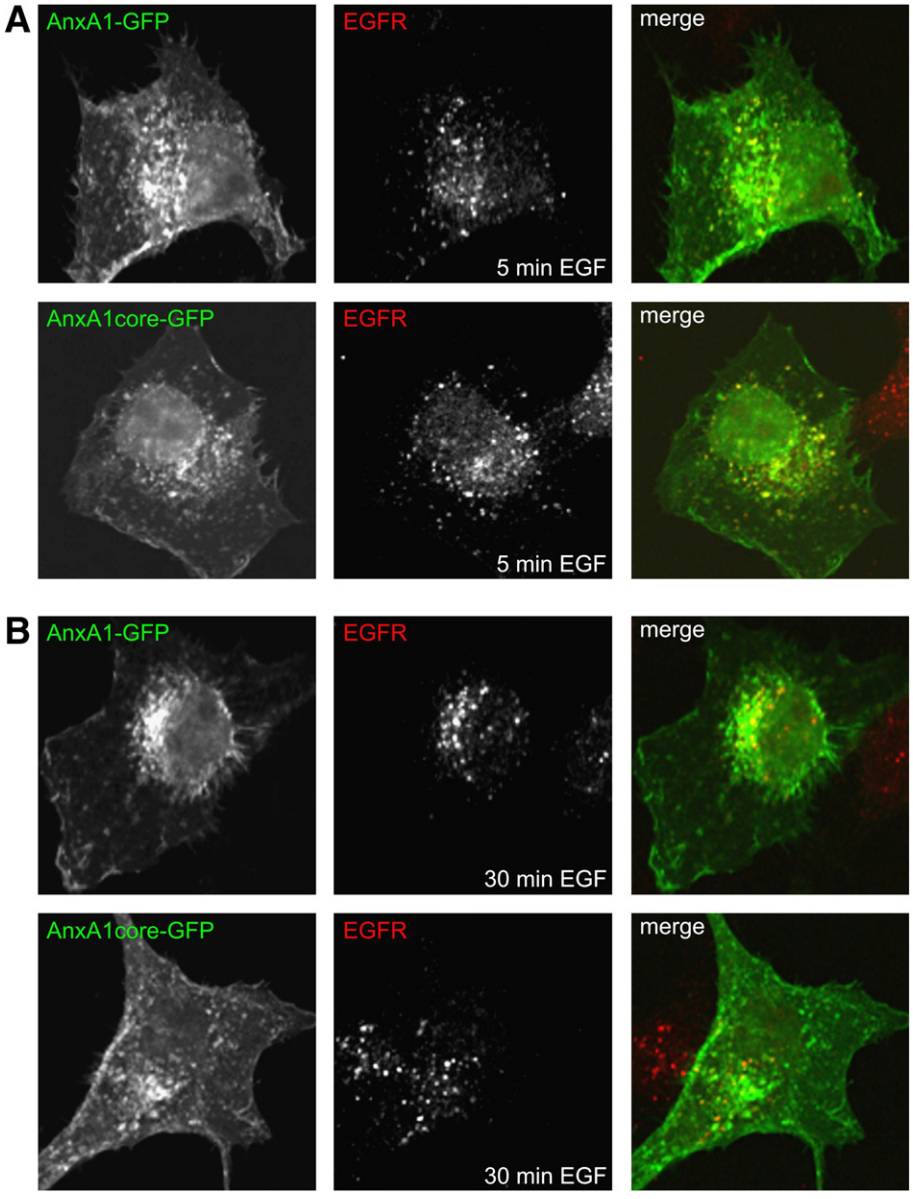


Fig. 3. EGFR transport and signal termination is impaired in cells expressing the AnxA1 core domain. Serum-starved HeLa cells expressing AnxA1–GFP or AnxA1core–GFP were stimulated with EGF for either (A) 5 min or (B) 30 min and then immunostained with anti-EGFR antibodies to visualize EGFR distribution. Note that EGFR positive endosomes appeared more dispersed in cells expressing AnxA1core–GFP upon 30 min of EGF stimulation. (C) Serum-starved HeLa cells expressing AnxA1–GFP or AnxA1core–GFP were treated with 100 ng/ml EGF in the presence of cycloheximide for the indicated periods of time. Degradation of EGFR was analyzed by immunoblotting cell lysates with anti-EGFR antibodies. A representative immunoblot from five independent experiments is shown. In addition to the 180 kDa band corresponding to mature EGFR, intermediate EGFR cleavage products are detected. Actin was used as a control to ensure equal loading of total cellular proteins. Kinetics of EGFR degradation was assessed by densitometric quantification of the mature EGFR, and expressed as the percentage of the EGFR level of cells at 0 min. Data represent mean values ± SEM. * indicates a statistically significant difference ($P \leq 0.05$). (D) Serum-starved A431 cells left untreated (control) or expressing either AnxA1–GFP or AnxA1core–GFP were treated with 10 ng/ml EGF for 30 min. Levels of EGFR and the MAP kinases MEK1/2 and their corresponding activation states were assessed by immunoblotting the cell lysates with the respective antibodies. A representative blot is shown.

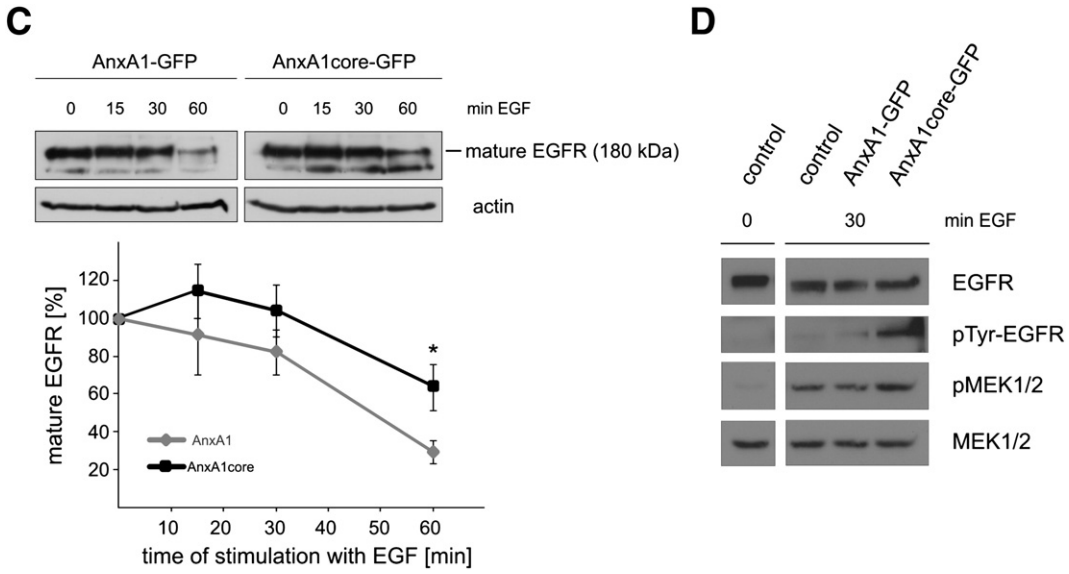


Fig. 3 (*continued*).

A1 N-terminal cleavage, thereby generating an annexin A1 core that cannot interact with S100A11 and has been discussed to participate in the MVB inward vesiculation process [5]. However, the exact cleavage site is not known. Therefore, we used an N-terminal deletion mutant which encompasses all possible cleavage products to study the potential involvement of the AnxA1/S100A11 complex in EGFR internalization. HeLa cells were transfected with AnxA1full-length–GFP or AnxA1core–GFP and the internalization and endocytic trafficking of fluorescently labeled EGF was followed using a pulse-chase regime. Texas Red-conjugated EGF was first allowed to internalize into transfected HeLa cells for 10 min. In control cells expressing GFP or AnxA1full-length–GFP a general cytosolic distribution of EGF-containing vesicles was detectable.

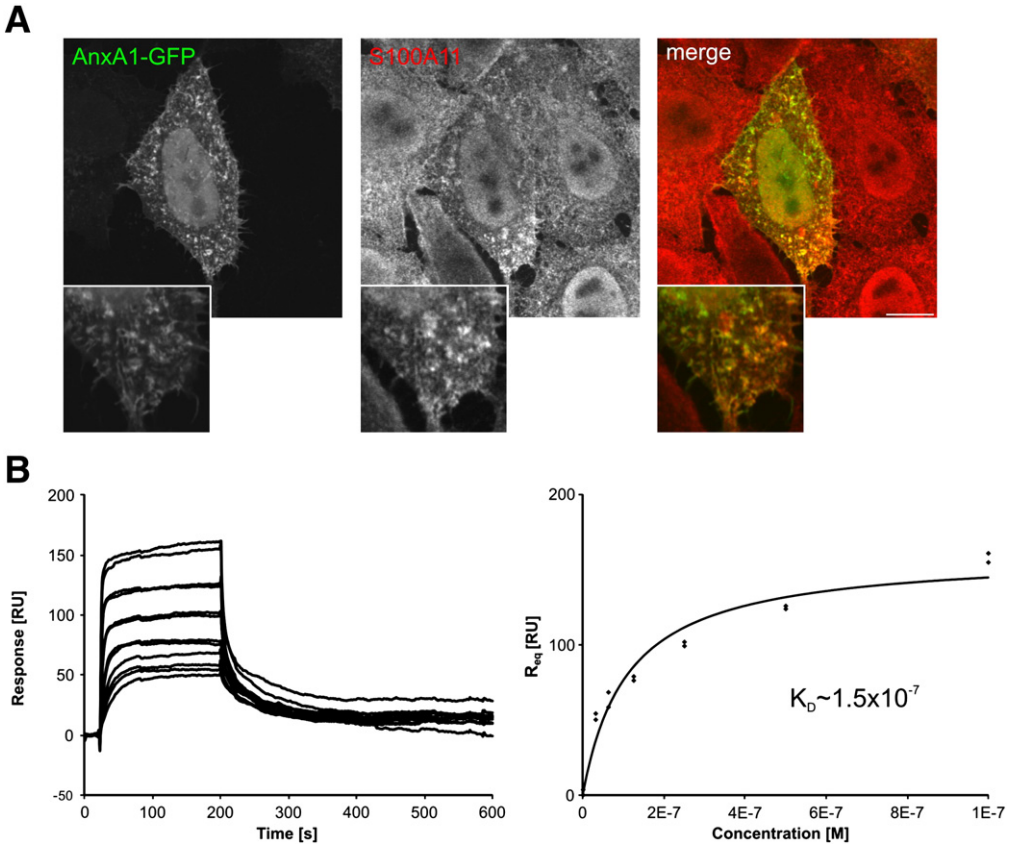


Fig. 4. Interaction of AnxA1 with S100A11. (A) HeLa cells expressing AnxA1–GFP were immunostained for the localization of endogenous S100A11. A representative image of a confocal section is shown. Boxed inserts show high magnification views of selected cell regions. Bar, 10 μm. (B) The affinity for S100A11 binding to AnxA1 was determined in vitro utilizing Surface Plasmon Resonance. Purified S100A11 (0.03125–1 μM) was passed over immobilized AnxA1 in the presence of 1 mM Ca^{2+} and the response units (RU) were plotted against time. Steady state affinity was calculated using the response at equilibrium (R_{eq}) at the end of injection as function of the sample concentration (M) and revealed a K_D value of ~1.5×10^{-7}.

EGF-containing vesicles in cells expressing the N-terminally truncated mutant appeared more peripherally enriched (Fig. 1A), possibly reflecting a delay in transport. After an additional incubation for 15 min, EGF-containing vesicles already clustered in the perinuclear region in cells expressing full-length AnxA1 and in GFP-expressing control cells. In contrast, labeled EGF was found in dispersed vesicular structures in the AnxA1core–GFP expressing cells (Fig. 1B). A similar delay in EGF-transport to the perinuclear region could be observed in A431 squamous carcinoma cells expressing the AnxA1 core mutant (our unpublished data).

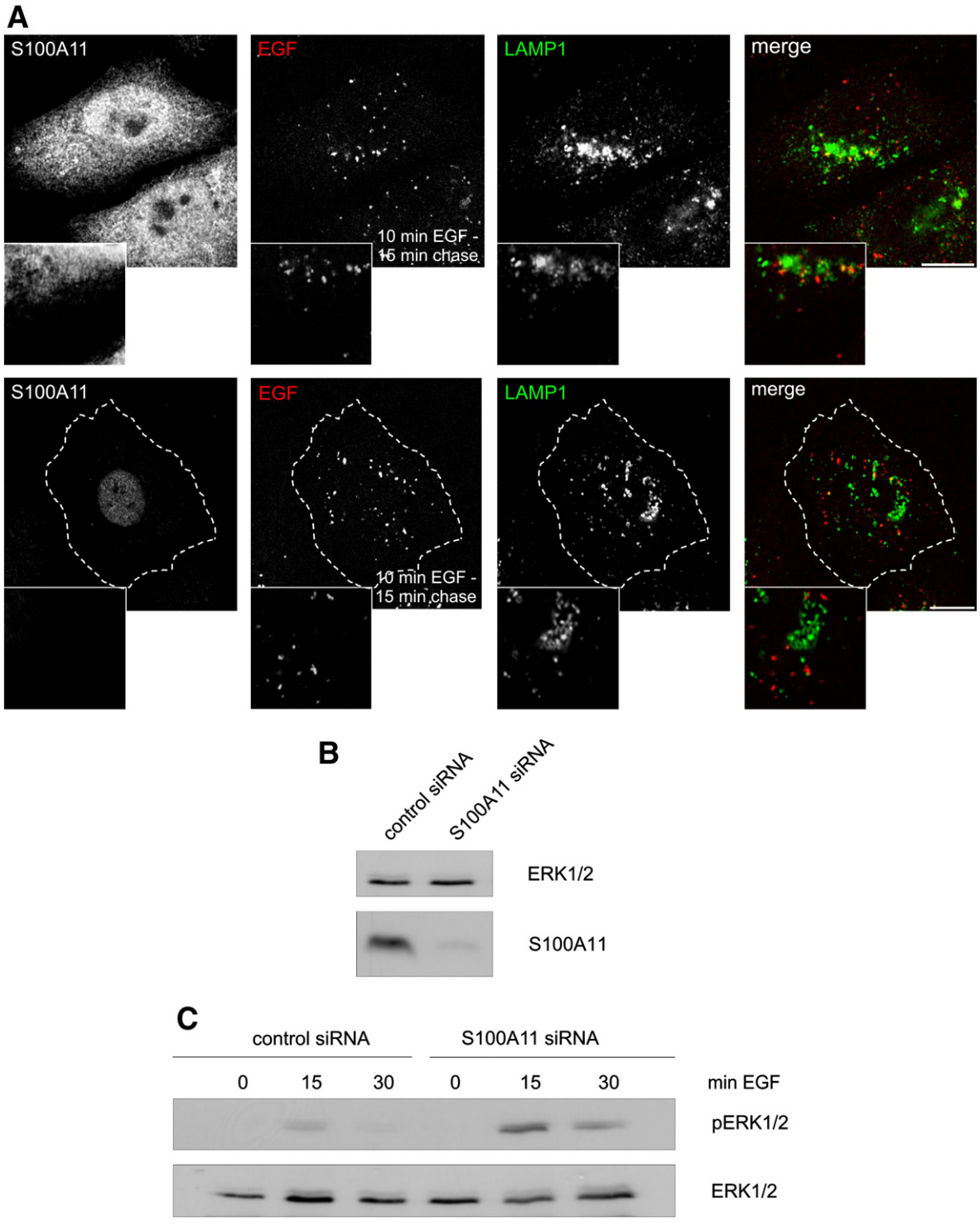
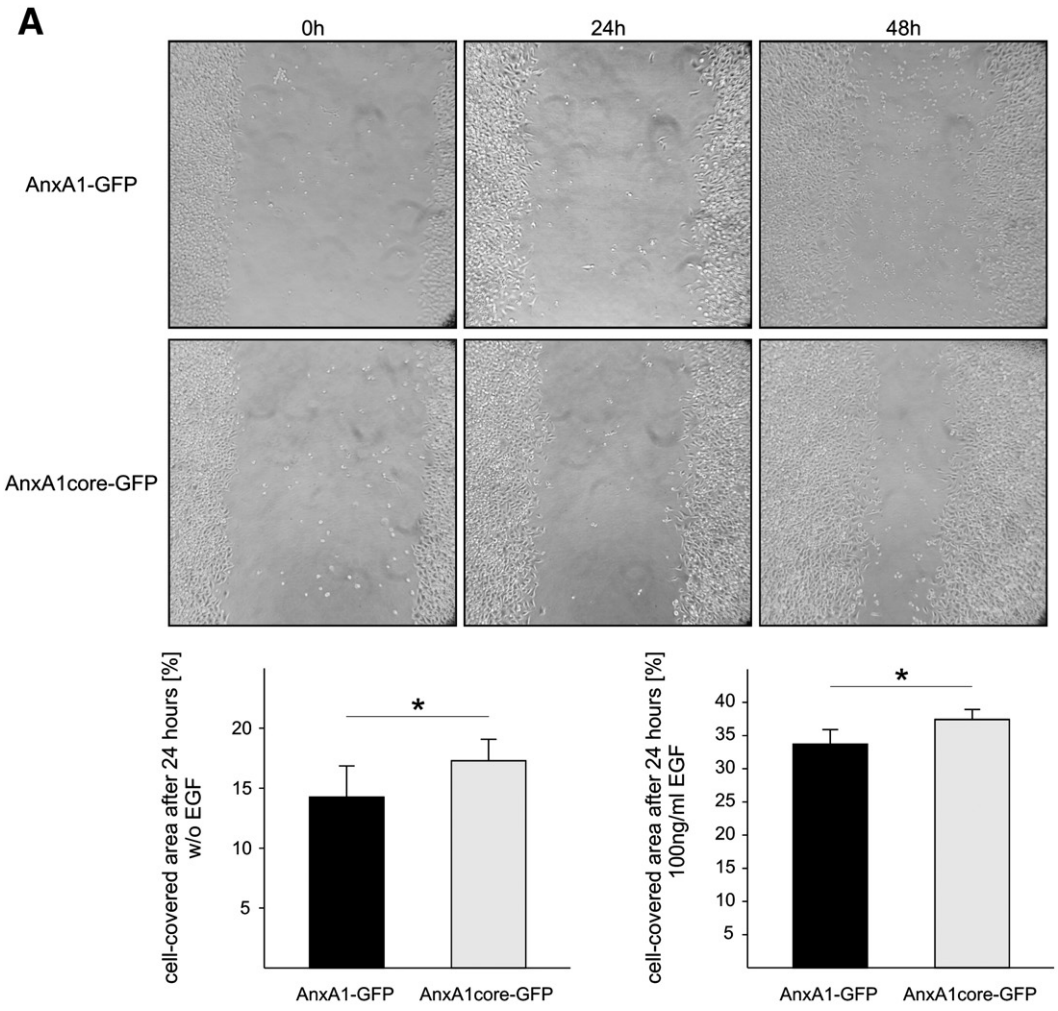


Fig. 5. S100A11 depletion results in impaired EGF transport and sustained EGF-induced signaling. (A) HeLa cells transfected with either control siRNA (upper panel) or S100A11 siRNA (lower panel) were serum-starved overnight. Texas Red-coupled EGF was internalized for 10 min, followed by a 15 min chase period. Fixed cells were stained for LAMP1. Images represent single confocal sections. Individual outlines of S100A11-depleted cells are indicated with dashed white lines. Boxed inserts show high magnification views of selected cell regions. Bars, 10 μm. (B) Total levels of the MAP kinases ERK1/2 and S100A11 in the lysates from cells transfected with control siRNA or S100A11-specific siRNA were detected with anti-ERK and anti-S100A11 antibodies, respectively. (C) Cells transfected for 48 hours with control siRNA or S100A11-specific siRNA were serum-starved overnight and then treated with 100 ng/ml EGF for the indicated times. ERK1/2 levels and activation profile was assessed by Western blot analysis.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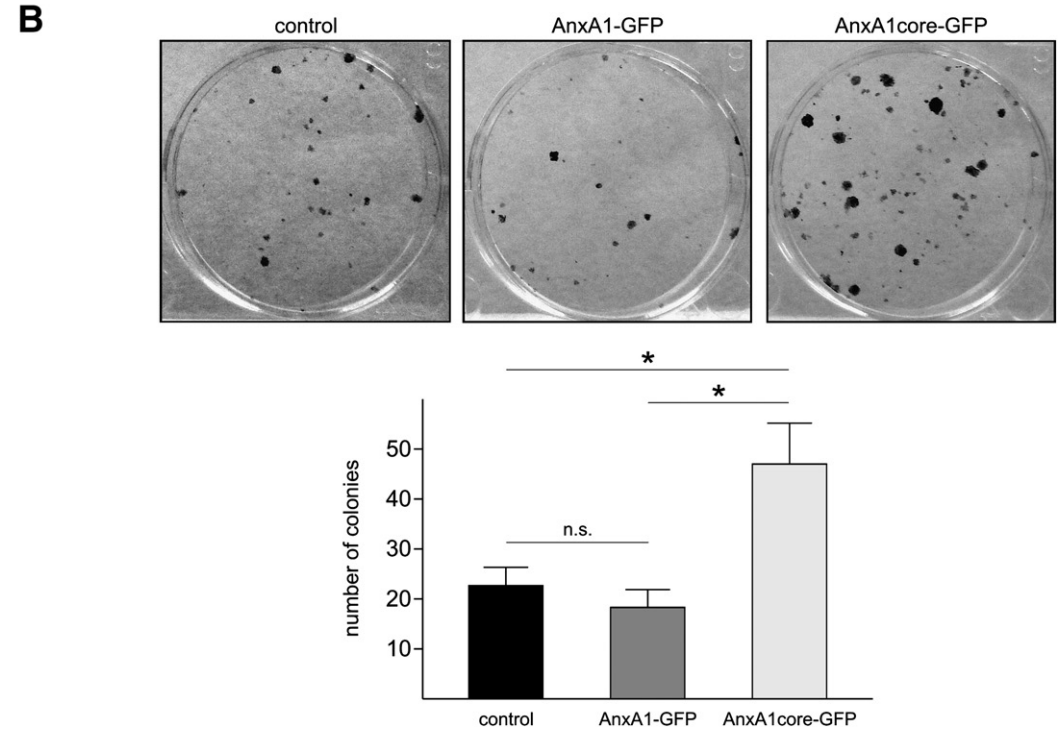
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To identify if the dispersed distribution of EGF-containing vesicles in N-terminal AnxA1 core mutant expressing cells would correlate with reduced amounts of EGF that can reach the lysosomal compartment, we analyzed the degree of colocalization of internalized labeled EGF with LAMP1, a marker for the late endosomal/lysosomal compartment in AnxA1–GFP cells and cells expressing AnxA1core–GFP. No difference in the amount of EGF colocalizing with lysosomes was observed between cells expressing full-length AnxA1 and AnxA1core–GFP expressing cells after the initial stimulation of 10 min. However, when internalized EGF was chased for 15 min a substantial amount of EGF could be found in LAMP1-positive endosomes in cells expressing full-length AnxA1, whereas in AnxA1core–GFP expressing cells this amount was significantly reduced, indicating a transitory delay in earlier endocytic transport steps along the degradative pathway (Fig. 1C). Although colocalization of LAMP1 and EGF was similar in cells expressing full length or the AnxA1core–GFP mutant after a chase period of 30 min, these findings suggested a delayed transport of EGF to the LAMP1-positive compartment upon overexpression of the N-terminal AnxA1 mutant.

To further validate these findings, we hypothesized that a different pathway emerging from the early endosomes, the recycling pathway back to the plasma membrane, which is typically taken by the transferring receptor, should not be affected. Therefore, AnxA1–GFP and AnxA1core–GFP expressing cells were continuously incubated with fluorescently labeled transferrin for 30 min. At this time all TfR-positive vesicles represent either early or recycling endosomes. As shown in Fig. 2A, no alterations in the distribution of the endosomal pool containing labeled transferrin could be observed between AnxA1–GFP and Anx1core–GFP expressing cells.

We previously demonstrated that endogenous and ectopically expressed full length AnxA1 localized on early endosomal membranes, while the AnxA1 core mutant lacking the N-terminal domain was associated with late endosomal structures. The difference in the target membrane preferences is not affected by the GFP tag [21,32]. To address if the delayed transport of EGF toward the late endosomal compartment in AnxA1core–GFP expressing cells would be associated with a different location of wild type and mutant AnxA1, we compared their localization utilizing subcellular fractionation on sucrose gradients. Early and late endosomal fractions were isolated by their different flotation behavior [31] and consistent with published data, the early endosomal marker Rab5 co-purified with early endosomes (EE) and was only faintly observed in the late endosomal (LE) fractions (Fig. 2B). To assess the enrichment, equal protein amounts of the corresponding EE and LE fractions were immunoblotted using anti-GFP antibodies and the LE/EE ratios were calculated from the densitometric analysis of the respective AnxA1 fusion protein. In contrast to AnxA1–GFP, AnxA1core–GFP was found significantly enriched in the late endosomal fraction. This is consistent with previous findings that the N-terminal part of AnxA1 is essential for directing the protein preferentially to early endosomes.

3.2. Delayed EGFR trafficking is linked to prolonged EGFR signaling

To identify if the delay in EGF transport in AnxA1core–GFP expressing cells was associated with impaired trafficking of the EGFR, HeLa cells expressing either AnxA1–GFP or AnxA1core–GFP were serum-starved over night and then stimulated with EGF for 5 or 30 min. Whereas EGFR localization in wild type and mutant AnxA1 expressing cells was comparable upon 5 min EGF incubation (Fig. 3A), indirect immunofluorescence utilizing anti-EGFR antibodies revealed that after 30 min of EGF treatment, the EGFR-positive compartment appeared more dispersed in the AnxA1core–GFP expressing cells (Fig. 3B), further supporting the view that transport along the endosomal pathway is affected in AnxA1core-expressing cells.

Depletion of AnxA1 has been shown to impair EGF-induced inward vesiculation in MVBs [9]. Since such impairment should affect the degradation of internalized EGFR, we investigated a possible role of the N-terminal domain of AnxA1 in the ligand-induced degradation of EGFR. HeLa cells transfected with either AnxA1full-length–GFP or AnxA1core–GFP were serum-starved to promote EGFR accumulation at the plasma membrane and then stimulated for 0–60 min with EGF in the presence of cycloheximide in order to inhibit new protein synthesis. Analysis of EGFR levels in total cell lysates by immunoblotting revealed that following 60 min of EGF stimulation the amount of mature EGFR was significantly decreased in control cells expressing AnxA1full-length–GFP. In contrast, lysates of cells expressing AnxA1core–GFP still contained a substantial amount of mature EGFR at 60 min after stimulation (Fig. 3C). Similar results were obtained for EGF-stimulated degradation of the EGFR in A431 cells (data not shown). Collectively, impaired EGFR degradation and delayed EGF-transport in cells expressing the AnxA1 core mutant suggest that loss of AnxA1/S100A11 interaction could affect early to late endosomal transport of endocytosed EGFR.

As EGFR degradation is closely coupled to the termination of EGF signal transduction, we also analyzed the activation of EGFR and the downstream signaling kinase MEK1/2 in mutant AnxA1 expressing cells. A431 cells were chosen for these experiments since they express high levels of EGFR [33]. A431 cells were transfected with AnxA1–GFP or AnxA1core–GFP, incubated with EGF for 30 min and EGFR tyrosine phosphorylation and MEK1/2 phosphorylation was determined. At this time point, substantial amounts of EGFR are already degraded in EGF-treated A431 cells [34]. Compared to untransfected control or AnxA1full-length–GFP expressing cells a markedly prolonged EGF-stimulated activation of the EGFR was observed in cells expressing the AnxA1 core mutant (Fig. 3D). Consistent with enhanced EGFR tyrosine phosphorylation, EGF-stimulated activation of MEK1/2 was also increased in AnxA1core–GFP overexpressing cells (Fig. 3D). Taken together, these findings indicate that activated EGFR is not targeted efficiently to late endosomes/lysosomes in AnxA1core–GFP expressing cells, thereby causing enhanced and prolonged signaling from EGFR and downstream effector pathways.

3.3. Depletion of S100A11 impairs transport of EGF

To address if AnxA1/S100A11 interaction could be involved in the lysosomal targeting of EGFR within endosomal compartments and possibly MVBs, we first aimed to confirm that AnxA1 is able to target the binding partner S100A11 to early endosomal membranes [29]. In line with previous observations, endogenous S100A11 clearly colocalized with full-length AnxA1–GFP in unstimulated HeLa cells (Fig. 4A). For a further characterization of the binding properties of AnxA1 and S100A11, we employed SPR to obtain quantitative information on the affinity of this interaction. Purified AnxA1 was immobilized on a CM5 sensor chip, and increasing concentrations of purified S100A11 were injected in the presence of 1 mM Ca^{2+}. Analysis of the data following the steady state affinity model revealed a K_D of ~1.5×10^{-7} for the interaction of S100A11 with AnxA1 (Fig. 4B). To provide evidence that AnxA1/S100A11 complex formation is needed for trafficking of ligand-activated EGFR, we next analyzed whether S100A11 has a role in EGF

Fig. 6. AnxA1core expression enhances migratory potential and clonogenic growth. (A) Cell migration assays were performed with HeLa cells expressing AnxA1–GFP or AnxA1core–GFP in the presence or absence (w/o) of EGF (100 ng/ml). After 24 hours, the cell-covered area in each well was calculated as percentage of the total well area. The graph shows the mean values ± SEM from at least four independent experiments. Statistical analysis was performed using Student's *t*-test. $^*P \leq 0.05$. Upper panel: Representative results of cells grown with EGF at indicated time points. (B) Clonogenic survival of A431 cells expressing AnxA1–GFP or AnxA1core–GFP. Clonogenic assays were performed with untransfected control cells, cells expressing AnxA1–GFP and AnxA1core–GFP expressing cells. After 5 days, numbers of colonies consisting of ≥40 cells were determined. Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison test. $^*P \leq 0.05$. The upper panel shows a representative result of four independent experiments.

transport and degradation. Therefore, fluorescently labeled EGF was internalized into serum-starved HeLa cells depleted for S100A11 and control siRNA transfected cells for 10 min incubation followed by an additional 15 min chase. While clustering of EGF-containing endosomal structures could be observed in control siRNA transfected cells (Fig. 5A, upper panel), EGF-containing vesicles were more dispersed in cells depleted of S100A11 (Fig. 5A, lower panel, compare enlarged areas in Fig. 5A). These results indicate that S100A11 is indeed important for EGF transport along the degradative pathway. We reasoned that if S100A11 acted together with AnxA1 on EGFR trafficking, the delayed transport of EGF should give rise to enhanced activation of the MAPKinase signaling cascade, similar to the findings observed in AnxA1core-expressing cells (see Fig. 3D). We first confirmed by immunoblotting that treatment of cells with the target-specific siRNA almost eliminated S100A11 expression, but had no impact on ERK1/2 expression (Fig. 5B). Next, the effect of S100A11 knock down on EGF-stimulated ERK activation was determined by Western blotting for phosphorylated ERK1/2. As shown in Fig. 5C, suppression of S100A11 expression prolonged the ERK activation profile, further supporting a model that S100A11, through its interaction with AnxA1, regulates the endosomal trafficking of EGFR.

3.4. AnxA1core expression affects EGF-induced cell migration and cell growth

To investigate if prolonged EGFR activation and MAP kinase signaling upon disturbing AnxA1/S100 complex formation were of any physiological significance and possibly relevant in the development or progression of cancer, we first investigated the migration behaviour of HeLa cells expressing either AnxA1–GFP or AnxA1core–GFP. Indeed, cells that had been transfected with the AnxA1–GFP construct could not migrate as efficiently as cells expressing AnxA1core–GFP (Fig. 6A). This difference was most robustly observed 24 hours after initiation of the migration.

We next employed the clonogenic assay as a tool to test whether AnxA1core–GFP-induced prolonged EGF-activated MAP kinase signaling had any effects on the survival and proliferation of cells. Upon plating, A431 cells were maintained for 10 days in EGF containing media and the obtained clones were then stained and counted. Whereas expression of AnxA1–GFP did not change the number of clones compared to cells expressing GFP only, more clones derived from AnxA1core-expressing cells were determined (Fig. 6B). Statistical analysis revealed that expression of AnxA1core was indeed associated with a significant increase in clone numbers.

4. Discussion

Stimulation of cells with EGF results in clustering of the receptor tyrosine kinase EGFR with its bound ligand and subsequent internalization into endocytic vesicles [35]. Activated and signaling competent EGFR is then sorted into early endosomes, sequestered into intraluminal vesicles of MVBs and ultimately degraded in the lysosomal compartment. Tight spatio-temporal regulation of the multitude of transport steps is essential for accurate termination of EGF signaling. Here we demonstrate the requirement of the N-terminal domain of AnxA1 in transport steps that result in the sequestration of internalized EGFR into the internal vesicles of MVBs. Thus, AnxA1 could function in the silencing of activated EGFR and hence in the regulation of mitogenic signals that also play a role in certain tumor developments. EGFR is implicated in a major fraction of human cancers and deregulation of AnxA1 expression has been reported in numerous cancer types [18]. Especially in breast cancer progression, down-regulation of AnxA1 [19] seems to correlate with up-regulation of the EGFR and its downstream effector pathways [20]. As AnxA1 is required for efficient EGFR degradation, down-regulation of AnxA1 in tumorigenesis may contribute to tumor pathology by potentiating EGF signaling.

Previous reports suggested that loss or depletion of AnxA1 did not significantly alter EGF degradation and only caused a small delay in EGFR degradation [9]. In the present study the employment of an N-terminally truncated AnxA1 mutant resulted in a significant delay in EGFR degradation. These differing results may be reconciled by the finding that the AnxA1 core protein redistributes from early to late endosomes when compared to the full-length AnxA1 protein [32,36]. Whereas depletion or loss of AnxA1 may result in compensatory mechanism possibly involving other annexin family members, the ectopically expressed AnxA1 core mutant might act in a dominant-negative manner and efficiently and specifically compete with endogenous AnxA1 for binding to late endosomes. In line with a reported role of AnxA1 in inward vesiculation in multivesicular endosomes [9], the specific replacement of endogenous AnxA1 by the core mutant may preclude functional redundancy within the annexin family and therefore delay EGFR transport and degradation more severely than the total lack of AnxA1.

As AnxA1 is present on internal vesicles of MVBs after EGF-stimulation [9] and associates with the EGFR via its core domain [36], we conclude that the AnxA1 core domain directly interacts with the EGFR during EGFR-transport along the endocytic pathway. The EGFR kinase phosphorylates AnxA1 on the tyrosine residue at position 21 [37–40] and this phosphorylation in turn has been demonstrated to be essential for EGF-stimulated AnxA1-dependent inward vesiculation in MVBs [9]. The delay in lysosomal EGFR degradation observed here upon expression of the AnxA1 core mutant correlates with the reported effects of N-terminal AnxA1 phosphorylation on both inward vesiculation and proteolytic degradation of AnxA1 and internalized EGFR. In line with these observations, an AnxA1 mutant deficient in the N-terminal phosphorylation site is not able to compensate for the effect of AnxA1 depletion on EGF-stimulated MVB biogenesis [9]. While such a phosphorylation deficient mutant is still able to exert functions that are mediated through interaction with its N-terminal binding partner S100A11 [25], the AnxA1 core mutant employed in this study also interferes with functions of a putative AnxA1-S100A1 complex. In this study, we could show that both ablation of S100A11 and overexpression of the AnxA1 core mutant delayed trafficking and degradation of the EGFR and interfered with termination of EGF-activated signaling, indicating that not only AnxA1 membrane binding but also complex formation with S100A11 is essential for EGF transport along the degradative pathway. As AnxA1 could exhibit membrane aggregating properties by formation of a heterotetrameric complex with S100A11 [41], such a complex could function in membrane fusion events during inward vesiculation along the degradative pathway. Based on our observations it is conceivable that the heterotetrameric complex of AnxA1 and S100A11 forms on the perimeter endosomal membrane during initial membrane invagination in nascent MVBs, thereby stabilizing membrane interactions at sites of emerging inward vesicles, for example by linking opposing membranes at the invaginating inward bud. Expression of an AnxA1 mutant lacking the N-terminal domain most likely results in the efficient replacement of endogenous AnxA1 during the passage of EGFR from early to late endosomes, thereby most likely inhibiting the postulated mechanism that couples AnxA1 to inward vesiculation in MVBs.

4.1. Concluding remarks

Endocytic transport of ligand-bound EGFR and receptor silencing by internal vesicle sequestration and ultimate degradation is a key event in regulating the duration of the downstream MAP kinase signaling cascade. We show that expression of the AnxA1 core mutant prolongs this signaling, supporting the view that the AnxA1/S100A11 complex participates in EGFR inward vesiculation and thereby the termination of EGF signaling. This directly couples the AnxA1/S100A11 complex to the spatio-temporal regulation of EGFR signal transduction. Upon activation, EGFR family members signal through MEK to ERK and cell migration and proliferation are among the processes regulated through activation of the ERK signaling cascade. Our observations that expression

of the AnxA1core mutant increased cell migration and enhanced the ability to form colonies from single cells emphasize the importance of the AnxA1/S100A11 complex formation to successfully balance EGF signaling. Since inappropriate signaling through EGFR/Ras/Raf/MAPK pathway is found in many tumors [42] these mechanistic insights may provide the molecular basis for the postulated involvement of S100A11 and AnxA1 in tumorigenesis.

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