Specific association of annexin 1 with plasma membrane-resident and internalized EGF receptors mediated through the protein core domain

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Abstract Phosphorylation of the Ca^{2+} and membrane-binding protein annexin 1 by epidermal growth factor (EGF) receptor tyrosine kinase has been thought to be involved in regulation of the EGF receptor trafficking. To elucidate the interaction of annexin 1 during EGF receptor internalization, we followed the distribution of annexin 1-GFP fusion proteins at sites of internalizing EGF receptors. The observed association of annexin 1 with EGF receptors was confirmed by immunoprecipitation. We found that this interaction was independent of a functional phosphorylation site in the annexin 1 N-terminal domain but mediated through the Ca^{2+} binding core domain.

Keywords: Annexin 1; Epidermal growth factor receptor; Endocytosis; Receptor clustering

1. Introduction

Annexin 1 is a member of the Ca^{2+} and phospholipid-binding annexin protein family of cytosolic proteins, which can reversibly and Ca^{2+}-dependently associate with cellular membranes. Its highly conserved protein core domain harbors the Ca^{2+}- and phospholipid-binding sites, whereas the N-terminal domain is variable and unique and is thought to regulate specific biological actions of annexin 1. Several annexins have been implicated in vesicular trafficking and membrane fusion events, and at least in some cases the Ca^{2+}-dependent membrane association of the proteins appears to be of critical importance for this role (for review, see [1]). Annexin 1 has been found to associate with the plasma membrane and endosomal membranes [2–6], and it has been shown that the integrity of the Ca^{2+}-binding sites is essential for the binding of annexin 1 to endosomal membranes [4,6]. The N-terminal domain of annexin 1 contains phosphorylation sites for protein kinase C and several tyrosine kinases including the epidermal growth factor (EGF) receptor tyrosine kinase, which phosphorylates tyrosine 21 [7–10]. Phosphorylation of the protein, which is induced by growth factors and cytokines, including EGF, insulin, HGF, growth hormone, and angiotensin II [2,11–14], is reported to participate in mediating or regulating the mitogenic response [15,16]. Annexin 1 is highly expressed and phosphorylated during hepatocarcinoma development and has been implicated in liver regeneration and tumorigenesis, probably by modulating EGF receptor (EGFR) activity [17]. A link between annexin 1 and EGFR signaling has also been observed in lung cells [18]. In particular, phosphorylation of annexin 1 by activated EGFR has been proposed to play a role in mediating inward vesiculation in multivesicular endosomes and thereby the sorting of internalized EGFR to the degradative lysosomal pathway [2]. Although a function of annexin 1 in regulating endosomal EGFR trafficking has long been postulated, an association of annexin 1 with EGFR during the course of receptor endocytosis has not been investigated so far. To analyze such spatial and temporal relation between EGFR and annexin 1, we therefore monitored the subcellular distribution of annexin 1 or annexin 1 mutant proteins labeled with green fluorescent protein (GFP) to sites of internalizing EGF receptors in single living cells. We show that antibody- clustered EGFR co-localizes with annexin 1 at the plasma membrane and at vesicular structures during internalization. This association, which can be verified by co-immunoprecipitation, requires the Ca^{2+}-binding annexin 1 core domain.

2. Materials and methods

2.1. Cell culture and transfection

The modified HeLa cell line HeLa dyn^{K44A} expressing a non-functional dynamin mutant under tetracycline control [19] was kindly donated by Dr. S. Schmid (The Scripps Research Institute, La Jolla, USA). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) supplemented with 5% fetal calf serum, glutamine and 1 μg/ml tetracycline (Sigma–Aldrich, Taufkirchen, Germany) in a 5% CO_2 incubator at 37 °C. The overexpression of the non-functional, dominant-negative dynamin was induced by tetracycline deprivation for at least 48 h. For transient transfection, the calcium phosphate method was used. Prior to transfection, the cell culture medium was replaced with fresh medium. 1–3 μg DNA was diluted in 150 ml HEPES buffer (HBS: 20 mM HEPES, pH 7.0, 140 mM

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor

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NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4 and 0.1% glycerol) and mixed with 10 μl of a 2 M $CaCl_2$ solution. The mixture was incubated at room temperature for 30 min and added to the culture medium of one well of a 6-well plate. Cells were used for experiments 24 h after transfection.

2.2. Antibodies and immunoblotting

The rabbit polyclonal anti-annexin 1 antibody r656 was described previously [4]. Rabbit polyclonal anti-EGFR antibody 1005, mouse monoclonal antibody 528 recognizing a cell surface epitope of human EGFR, and mouse monoclonal anti-GFP antibody were purchased from Santa Cruz Biotechnology Inc. (California, USA). Total cell lysates or immunoprecipitated proteins were resolved by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose. The membrane was blocked with 5% non-fat milk powder in TBS-T (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% Tween 20) prior to probing with the primary antibody for 1 h at room temperature and then with the HRP-coupled appropriate secondary antibodies (Amersham Pharmacia Biotec, Freiburg, Germany). Bands were visualized by ECL (Amersham Pharmacia Biotec, Freiburg, Germany).

2.3. Annexin 1 expression constructs

The expression constructs anx1-GFP, cmanx1-GFP, and anx1core-GFP were described previously [1]. To generate the plasmids encoding anx1Y21A-GFP and anx1Y21E-GFP with a mutated EGFR phosphorylation site Y21, a fragment encoding amino acids 1–27 of the annexin 1 N-terminal domain was excised from wild-type anx1-GFP and then replaced with synthetic double-stranded oligonucleotides installing the amino acid exchanges Y21A or Y21E. Constructs were confirmed by sequencing (SEQLAB, Göttingen, Germany).

2.4. Preparation of cell lysates and immunoprecipitation

The cell monolayers were rinsed with ice-cold PBS and then lysed for 30 min with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% DOC, and 1% NP-40) containing a cocktail of protease and phosphatase inhibitors (10 μg/ml leupeptin, 10 μg/ml E64, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 40 μl/ml trypsin inhibitor, 4 mM AEBSF, 10 μM benzamidin and MG-101, 50 mM NaF, 10 μM $NaVO_3$, 2 mM sodium pyrophosphate and 10 mM β-glycerophosphate). Lysates were centrifuged for 20 min at 20 000 × *g*. Supernatants were incubated at 4 °C with 4 μg primary antibody for at least 2 h and subsequently incubated for two hours with protein A–Sepharose (Amersham Pharmacia Biotec, Freiburg, Germany). Beads were spun down, washed three times with RIPA buffer and the adsorbed proteins were subjected to immunoblotting analysis.

2.5. In vivo labeling of EGF receptor and EGFR clustering

Transfected cells grown on coverslips were serum-starved overnight. For EGF receptor labeling with fluorescent EGF, cells were exposed to EGF complexed to Texas Red® (Texas Red® EGFcomplex, Molecular Probes, Leiden, The Netherlands) at a final concentration of 2 μg/ml in serum-free medium containing 1% BSA at 37 °C. For EGFR clustering, cells were incubated with anti EGFR antibody 528 directed against a cell surface epitope of human EGFR (diluted 1:100 in serum-free Dulbecco's modified Eagle's medium containing 1% BSA) for 15 min at 37 °C. Subsequently, cells were rinsed with PBS and clustering of EGFR was obtained by subsequent incubation with Texas Red®-labeled secondary antibodies (15 μg/ml) for 15 min at 37 °C. The cells were then rinsed with ice-cold PBS and fixed with 4% PFA for 15 min at room temperature. In control cells, fixation was carried out after incubation with the primary antibody, i.e., prior to receptor clustering. Coverslips were mounted in Mowiol containing 4% *n*-propyl gallate as antifade agent and analyzed using a confocal laser scanning microscope (Zeiss LSM 510 Meta).

3. Results and discussion

3.1. Annexin 1 co-localizes with internalizing EGFR

In order to analyze the spatial and temporal association of annexin 1 with EGFR, we employed HeLa cells ectopically expressing annexin 1-GFP, which was shown previously to localize to the same structures as endogenous annexin 1 [6]. Consistent with previous observations, the annexin 1-GFP signal was present on vesicular structures dispersed throughout the cell which had been identified earlier as transferrin receptor-positive endosomes [6]. Upon incubation of annexin 1-GFP expressing cells with fluorescent Texas Red® EGF complex for 5 min at 37 °C, co-localization of internalized EGF with annexin 1 could be observed (Fig. 1A, upper panel). To analyze whether annexin 1 also associates with EGF-bound EGFR at the plasma membrane or only localizes to intracellular vesicles containing internalized receptor, we used HeLa cells stably transfected with dyn^{K44A}, which are conditionally defective for receptor-mediated endocytosis. In this cell line overexpressing a dominant-negative GTPase-deficient mutant dynamin (dyn^{K44A}), endocytosis of ligands such as transferrin and EGF is effectively blocked, probably by inhibiting the fission of clathrin-coated vesicles from the plasma membrane [19–21]. As shown in Fig. 1A, we observed a marked reduction of intracellular vesicles containing annexin 1 and internalized EGF in the dyn^{K44A} overexpressing cells. Rather enhanced annexin 1-GFP and Texas Red® signals are observed at the plasma membrane of the dyn^{K44A} overexpressing cells (Fig. 1A, lower panel).

To further enhance the EGFR label at the plasma membrane, we next used anti-EGFR antibodies to induce receptor oligomerization and selectively cluster the plasma membrane resident receptor into larger structures which are easily identified by microscopy. Serum-starved cells were incubated for 15 min with an antibody directed against the external domain of the EGFR that competes with EGF for the EGF-binding site and causes receptor dimerization [22]. The cells were then directly, without fixation or any other treatment, incubated with a secondary antibody linked to a red fluorescent dye. Thereafter, the cells were fixed with PFA. In controls, cells were fixed between the antibody incubations to prevent clustering. In these control cells, EGFR was diffusely distributed over the plasma membrane. In contrast, the antibody cross-linking in live cells led to the formation of EGFR clusters (Fig. 1B). Interestingly, these clusters co-localized with the annexin 1-GFP-positive vesicles. As seen in the *xz* sections, some of the clusters were internalized into the cell. However, co-localization of the EGFR clusters was not observed with annexin 3- or annexin 6-GFP (not shown).

Next, we examined whether plasma membrane attachment of vesicles containing EGFR clusters is a prerequisite for annexin 1 recruitment. Therefore, we performed in vivo crosslinking of EGFR in HeLa cells induced to express dyn^{K44A}. As shown in Fig. 1B, induction of dyn^{K44A} expression by tetracycline deprivation results in an accumulation of EGFR clusters at the plasma membrane. Likewise, we also observed a shift of the annexin 1-GFP signal which was concentrated beneath the plasma membrane.

3.2. Association with EGFR is mediated by the annexin 1 core domain

The annexin 1 N-terminal domain harbors a phosphorylation site (Tyr21) for EGFR tyrosine kinase activity. Phosphorylation of the protein through the internalized receptor has been proposed to regulate inward vesiculation in late endosomal compartments during receptor internalization [2]. To analyze the contribution of this phosphorylation to the recruitment of annexin 1 to sites of EGFR clusters, we generated annexin 1 mutants in which Tyr21 had been replaced by Ala

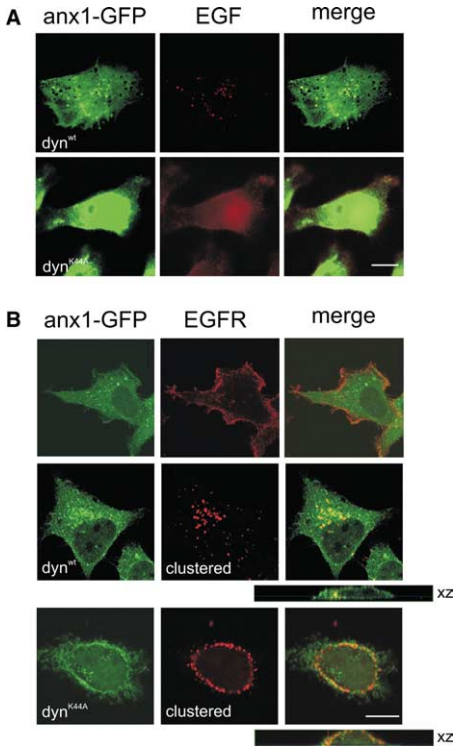

Fig. 1. Co-localization of annexin 1-GFP with EGFR. Non-induced HeLa cells (dyn^{wt}) or cells induced to express dominant-negative GTPase-deficient $dynamin^{K44A}$ (dyn^{K44A}) were transiently transfected with GFP-tagged annexin 1. (A) Cells were exposed for 5 min to Texas Red® EGF. In the absence of dyn^{K44A} (upper panels), fluorescent EGF was taken up into intracellular vesicles positive for annexin 1-GFP, whereas in cells expressing dyn^{K44A} (lower panels), both the EGF and the annexin 1-GFP signals accumulated at the cell periphery. Note that for obtaining higher signal intensities to better visualize the EGF and annexin 1-GFP distributions, the images were obtained by epifluorescence microscopy. (B) In vivo antibody labeling of EGFR was either followed by cross-linking of EGFR–antibody complexes with secondary antibodies (clustered) or by fixation and subsequent staining with secondary antibodies. Confocal x-y analysis of the EGFR distribution shows that after crosslinking of EGFR, the receptor accumulated in clusters at the plasma membrane whereas in cells treated with anti-EGFR antibodies followed by immediate fixation, EGFR was diffusely distributed. Confocal x-z sections reveal that in wild-type cells, clustered receptors appear to be internalized whereas in dyn^{K44A} expressing cells they remain at the plasma membrane. Bar, 10 μm.

(anx1Y21A-GFP) to prevent phosphorylation by activated EGFR or by Glu (anx1Y21E-GFP) to mimic a phosphorylated tyrosine residue. Interestingly, in each case, co-localization of the GFP fusion proteins with EGFR clusters was detected (Fig. 2), ruling out the possibility that phosphorylation at Tyr21 leads to a major change in the localization of annexin 1 at vesicles containing internalized receptor clusters. Association of annexin 1 with endosomes has been shown to be dependent on the integrity of the Ca^{2+}-binding sites in the core domain [6]. To elucidate whether the annexin 1 association with the EGFR clusters is also dependent on the Ca^{2+}-binding sites found in the annexin core module, we first carried out the EGFR clustering in cells expressing an N-terminally truncated annexin 1-GFP fusion protein which lacks the first 40 amino acid residues (anx1core). Again, association of this mutant with plasma membrane-resident and internalized EGFR clusters could be observed (Fig. 2). We next expressed the mutant cmanx1-GFP in which all three high affinity Ca^{2+}-

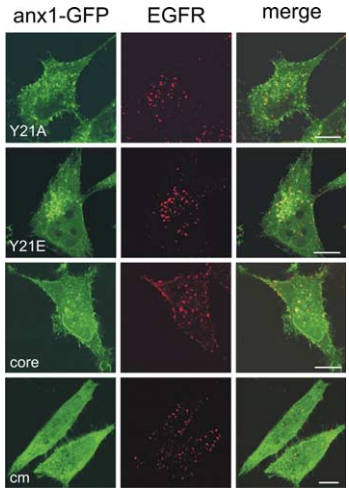

Fig. 2. EGFR was in vivo crosslinked in cells transiently expressing annexin 1-GFP fusion proteins as indicated. Annexin 1 mutants with Tyr21 replaced by Ala (anx1 Y21A-GFP) to prevent phosphorylation by activated EGFR or by Glu (anx1 Y21E-GFP) to mimic a phosphorylated tyrosine residue, or the N-terminally truncated annexin 1-GFP fusion protein lacking the first 40 amino acid residues (anx1core) localized to EGFR clusters. Cells expressing cmanx1-GFP (all three type II high affinity Ca^{2+}-binding sites inactivated) show a diffuse cytosolic GFP staining and no co-localization of the GFP label with EGFR clusters was observed. Bars, 10 μm.

binding sites, the so-called type II sites, were inactivated. Cells expressing this mutant show a diffuse cytosolic GFP staining as observed previously [1], and no co-localization of the GFP label with EGFR clusters or intracellular vesicular structures (Fig. 2). Taken together, these results indicate that the Ca^{2+}-dependent membrane binding mediated through the annexin 1 core is required for association of annexin 1 with the EGFR clusters. However, we cannot rule out the possibility that there are subtle differences in the endosomal populations to which the different annexin 1 mutants are directed.

To confirm the association of annexin 1 with the EGFR revealed by confocal fluorescence imaging of the annexin 1-GFP expressing cells, we carried out co-immunoprecipitation experiments using total cell lysates from HeLa cells transfected with the annexin 1-GFP expression plasmids. Equal amounts of proteins from the lysates were subjected to immunoprecipitation with antibodies against EGFR. In parallel, the presence of the annexin 1-GFP fusion proteins in the lysates was verified by analyzing samples of the lysates by Western blotting using anti-annexin 1 antibodies or anti-GFP antibodies. As shown in Fig. 3A, all chimeric proteins were expressed at a comparable level. Consistent with the results obtained by direct fluorescence microscopy, all of the different annexin 1 derivatives co-localizing with EGFR clusters, i.e., wt, truncated or tyrosine 21 mutants, were co-precipitated with the EGFR, whereas cmanx1-GFP was not detected in the immunoprecipitates (Fig. 3B). Hence, the site in annexin 1 required for association with EGFR is not localized in the N-terminal domain and phosphorylation of tyrosine-21 is not required for EGFR/annexin 1 interactions.

3.3. Conclusions

By in vivo labeling of EGFR on the cell surface of HeLa cells expressing annexin 1-GFP fusion proteins, we could show that annexin 1 co-localizes with the cross-linked receptor. The

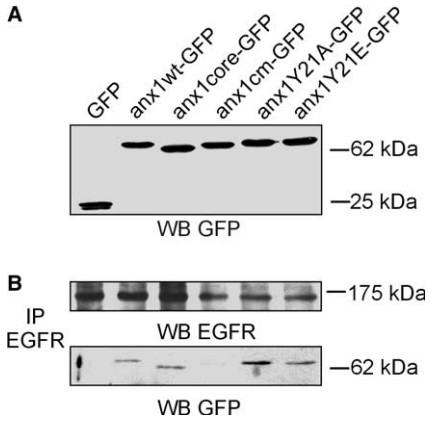

Fig. 3. Co-immunoprecipitation of EGFR with annexin 1-GFP derivatives. (A) Cell lysates from HeLa cells transfected with the indicated annexin 1-GFP plasmids or the empty vector as control were analyzed for expression of the respective fusion protein by SDS–PAGE and immunoblotting with anti-GFP antibody. (B) Subsequently, the lysates were subjected to immunoprecipitation (IP) with anti-EGFR antibody and the precipitated proteins were probed by immunoblotting (WB) with either anti-EGFR or anti-GFP antibody.

recruitment of annexin 1 to EGFR containing membrane structures already occurs at the plasma membrane, since it could be observed in cells where dynamin-dependent endocytosis is blocked. It is specific for annexin 1 and could not be observed with other annexins like annexin 3 and annexin 6. Mutations in the N-terminal Tyr21, the phosphorylation site for EGFR kinase activity, do not abrogate the co-localization of this mutant protein with the internalized receptor. Furthermore, the association is mediated by the core domain of annexin 1 and seems to depend on the integrity of the Ca^{2+}-binding sites of the protein.

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