Identification of 14-3-3ζ as an EGF receptor interacting protein

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Abstract The 14-3-3 proteins are known to interact with a number of proteins involved in the regulation of cell signaling. Here, we describe an association of 14-3-3ζ with the epidermal growth factor receptor (EGFR) that is rapidly induced by EGF. The 1028-EGFR truncated mutant which lacks the cytoplasmic tail from amino acids 1029–1186 identified the binding site for 14-3-3 to be between amino acid 1028 and the receptor carboxyl terminus. Mutational deletion of serine residues 1046, 1047, 1057 and 1142 did not inhibit EGF-induced 14-3-3 association with the receptor. Immunofluorescence microscopy indicated an EGF-induced co-localization of EGFR and HA-14-3-3ζ along the plasma membrane. Our finding adds to the growing complexity of EGF receptor signaling and indicates a role for 14-3-3 proteins in EGF receptor signaling or regulation.

Keywords: Receptor tyrosine kinase; Adapter protein; 14-3-3; Signal transduction

1. Introduction

The epidermal growth factor receptor (EGFR) belongs to the large family of cell surface receptors with intrinsic tyrosine kinase activity. The 175 kDa receptor is composed of an extracellular ligand binding domain, a single membrane spanning region, and an intracellular tail containing the tyrosine kinase domain and multiple binding sites for signaling molecules [1]. Following binding of EGF, the receptor dimerizes, autophosphorylates, and then nucleates signaling complexes. Numerous substrates of EGFR have been identified, including Grb2 [2], Shc [3], c-Cbl [4], PLCγ1 [5], and Gab1 [6].

The 14-3-3 family of proteins are ubiquitously expressed and involved in the regulation of diverse intracellular processes of several signaling proteins in all eukaryotic organisms [7]. Important 14-3-3-mediated activities include regulation of cell cycle arrest, intracellular signaling in response to stress, photoreceptor development and learning in *Drosophila*, and signaling from Ras/Raf [9,11–13]. There are seven known mammalian 14-3-3 isoforms (β, σ, ε, ζ, η, σ, and τ) with molecular masses of 28–33 kDa. The 14-3-3 proteins are evolutionary highly conserved from yeast to mammals and the different 14-3-3 isoforms display a high level of homology [8]. The 14-3-3 proteins are adaptor and scaffolding proteins that interact with proteins containing distinct phosphoserine/threonine motifs [10]. 14-3-3 can also form complexes with non-phosphorylated proteins [8]. In addition, two serine and one threonine phosphorylation sites on 14-3-3ζ have been identified [11]. 14-3-3 can regulate its binding partners by altering their intracellular localization, catalytic activity or complex formation with other proteins [9,14]. Here, we show that stimulation of cells with EGF induces binding of 14-3-3ζ to the EGFR carboxy-terminal tail, however the functional relevance of this complex formation remains to be determined.

2. Materials and methods

2.1. Materials

The affinity purified rabbit anti-14-3-3 antibody was a kind gift from M. Guthridge and A.F. Lopez. The antibody was raised in New Zealand White rabbits using glutathione *S*-transferase-14-3-3ζ (GST-14-3-3ζ) as the immunogen [15]. Purified human 14-3-3ζ was obtained from F.C. Stomski and A.F. Lopez. The expression vector for HA-tagged 14-3-3ζ (pEF/HA-14-3-3ζ) was obtained from R. Marais. A rabbit anti-14-3-3 antibody recognizing all 14-3-3 isoforms was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-HA (12CA5) was obtained from Roche (Indianapolis, IN). A sheep anti-EGFR antibody was purchased from Fitzgerald Industries (Concord, MA). The R42/pY1173 rabbit antisera are specific to phosphorylated Tyr1173 in EGFR and have previously been described [24]. Cy3-conjugated donkey anti-sheep and Cy2-conjugated donkey anti-mouse and peroxidase-conjugated donkey anti-mouse and anti-sheep antibodies were purchased from Jackson (West Grove, PA). Electrophoresis reagents were purchased from BioRad (Hercules, CA). Lipofectamine was obtained from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

2.2. Cell culture and transfection

The mouse fibroblast NIH3T3 cells were obtained from ATCC and cultured in DMEM (Trace Biochemicals) containing 10% FCS (Gibco-BRL) and 2 mM L-glutamine (Trace Biochemicals) at 37 °C and 5% CO_2. Cells were stably transfected with human full length EGFR by retroviral infection, as described earlier [16]. A truncated form of EGFR (1028-EGFR) was generated by site directed mutagenesis to introduce stop codons at amino acids 1029 of the human EGFR, and transfection by retroviral infection was performed as previously described [16]. The EGFR serine mutants (S1046A/S1047A and S1046A/S1047A/S1057A/S1142A) were expressed in the pLEN vector, as described earlier [17]. The vector control and the mutants were stably transfected by retroviral transfection into NIH3T3 cells under selection by G418 [16]. All constructs were examined by automated DNA sequencing (373 ABI).

2.3. Western immunoblotting and co-immunoprecipitation

Cells growing in Petri dishes were starved in 0.5% FCS overnight and incubated or not in 10 nM human recombinant EGF. Cells were

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rinsed in ice-cold PBS and lysed in Tris lysis buffer, pH 7.4 (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 20 mM NaF, and 1 μg/ml chymostatin, leupeptin and antipain). Lysates were incubated on ice for minimum 15 min, and cell debris and nuclei were removed by centrifugation. For analysis of total cell lysates, a 5× protein sample buffer stock solution (2% SDS, 10% glycerol, 0.02% bromophenol blue and 2% β-mercaptoethanol final concentrations) was mixed with the lysate. The samples were boiled for 5 min. For co-immunoprecipitation, 50 μl protein A–Sepharose (Amersham Biosciences, Uppsala, Sweden) was incubated with 2 μl monoclonal anti-EGFR (Santa Cruz Biotechnology) in 50 mM Tris, pH 7.2, for 2 h. Lysates were incubated with pre-washed protein A–Sepharose-anti-EGFR complex for 1 h on ice and washed three times with 25 mM Tris, pH 7.2. Samples were resuspended in 90 μl Laemmli sample buffer, pH 7.4 (10 mM Tris, 10% v/v glycerol, 2% w/v SDS, 5 mM EDTA, 0.02% bromophenol blue, and 2% β-mercaptoethanol), and boiled for 5 min. Samples were analyzed by SDS–PAGE with 6% or 10% acrylamide gels. Proteins were transferred semidry to nitrocellulose membranes, rinsed in ice-cold Tris-buffered saline [TBS; 10 mM Tris, pH 8.0, and 150 mM NaCl] and incubated in blocking buffer (TBS containing 5% dried milk, 1 mM NaF, and 1 mM Na_3VO_4) for 1 h at 4 °C. The membranes were incubated overnight at 4 °C with rabbit anti-14-3-3ζ (EB1), sheep anti-EGFR, or rabbit anti-pY1173 EGFR. Proteins were detected using HRP-conjugated secondary antibodies for 90 min at RT. All antibodies were diluted in TBS containing 1% w/v dry milk (Bonlac Foods, Melbourne, Australia) and 0.01% thimerosal. The filters were washed in TBS and antigens were visualized by the enhanced chemiluminescence (ECL) method with Hyperfilm MP (Amersham Biosciences).

2.4. Filter binding of 14-3-3ζ

Total cell lysates were separated by SDS–PAGE as described. Proteins were transferred to a nitrocellulose membrane and incubated with 5% dried milk in TBS with 1 mM Na_3VO_4 and 10 mM NaF for 30 min. The membrane was probed with 2 μg/ml 14-3-3ζ in the same solution at 4 °C for 4 h prior to an 8 × 2 min wash in TBS. The membrane was incubated with 14-3-3 antibodies (EB1) at 4 °C overnight and processed as described earlier for Western immunoblotting analysis.

2.5. Immunofluorescence microscopy

HeLa cells cultured in 60-mm plastic dishes were washed in PBS and fixed in ice-cold methanol for 4 min. Cells were incubated with primary antibodies overnight, followed by Cy2- and Cy3-conjugated donkey antibodies to IgG of the appropriate species. All dilutions were in PBS containing 1% BSA. Cells were studied in a Leica TCS SP confocal microscope equipped with an Ar (488 nm) and two He/Ne (543 and 633 nm) lasers. A Plan apochromat 100×/1.4 oil objective was used. Images were acquired sequentially.

3. Results and discussion

In order to identify interaction between the EGFR and members of the 14-3-3 protein family, EGFR was immunoprecipitated from HeLa cell lysates derived from cells that had been treated with or without EGF. Immunoblotting with a polyclonal anti-14-3-3 pan antibody specifically recognized a band at the appropriate molecular mass in cells stimulated with EGF (Fig. 1). The interaction was detected shortly after exposure to EGF (0.5 min). Analyses of cell extracts after immunoprecipitation of the EGFR by immunoblotting with the anti-14-3-3 pan antibody did not display any obvious differences in 14-3-3 levels in control cells and EGF-stimulated cells (data not shown). This indicates that the fraction of total 14-3-3 that is co-immunoprecipitated with the EGF-activated EGFR is low. From these results we hypothesized that EGFR and 14-3-3 form a complex in response to EGF-stimulation. Among the seven known mammalian 14-3-3 isoforms, we decided to study the 14-3-3ζ isoform and possible interaction with the EGFR in more detail. 14-3-3ζ binds to several

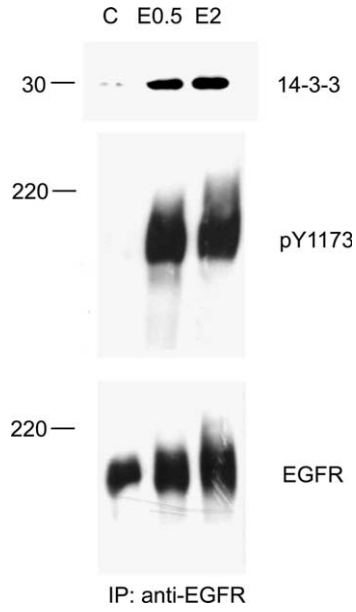

Fig. 1. Effect of EGF on 14-3-3 binding to the EGFR. EGFR immunoprecipitates from HeLa cells untreated (C) or stimulated with EGF (E) for 0.5 and 2 min were analyzed by Western immunoblotting. Rabbit anti-14-3-3 (A), rabbit anti-pY1173, and sheep anti-EGFR antibodies were used. The markers at left indicate 30 and 220 kDa.

different enzymes and has a role in the Ras-Raf-MEK-MAPK signaling pathway [9]. We tested whether the 14-3-3ζ isoform associates with the EGFR by filter binding. Total cell lysates from control and EGF-stimulated NIH3T3 cells overexpressing the human EGFR were separated by SDS–PAGE and transferred to filter. Following blocking, the filter was incubated with purified 14-3-3ζ protein for 4 h, before rinsing and incubation with the anti-14-3-3 pan antibody. The 14-3-3ζ protein was detected at a molecular weight corresponding to the EGFR in lysates from cells stimulated with EGF and not in unstimulated cells (Fig. 2). These findings indicate that the EGFR is capable of interacting directly with 14-3-3ζ.

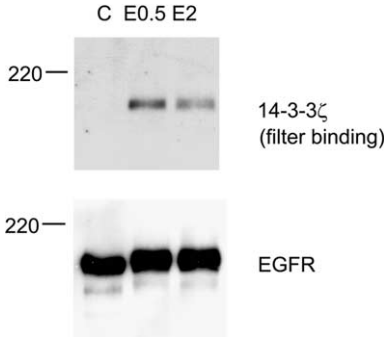

Fig. 2. Detection of 14-3-3ζ binding by filter binding. Total lysates from NIH3T3-EGFR cells untreated (C) or stimulated with EGF for 0.5 and 2 min were separated by SDS–PAGE and transferred to nitrocellulose filter. Following blocking, the filter was incubated with purified 14-3-3ζ protein (2 μM) for 4 h at 4 °C. The filter was rinsed in TBS and immunoblotted with an affinity purified rabbit anti-14-3-3 antibody. Total lysates were also analyzed with a sheep anti-EGFR antibody to demonstrate equal protein levels. The markers at left indicate 220 kDa.

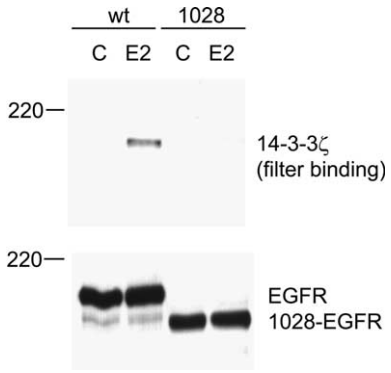

Fig. 3. Analysis of 14-3-3ζ binding to EGFR truncation mutants. Total lysates from NIH3T3-EGFR cells (wt) and NIH3T3 cells expressing the 1028-EGFR truncation mutant (1028) were examined by filter binding. Lysates from untreated cells (C) or cells stimulated with EGF for 2 min (E2) were separated by SDS–PAGE and transferred to nitrocellulose filter. The filter was analyzed by immunoblotting with a rabbit anti-14-3-3 antibody (top panel) and a sheep anti-EGFR antibody (bottom panel). The markers at left indicate 220 kDa.

To identify the 14-3-3 binding site on EGFR, complex formation on truncated receptors was examined. We took advantage of NIH3T3 cells stably expressing the human truncation mutant 1028-EGFR which has the carboxy-terminal residues 1029–1186 removed [16]. Anti-EGFR immunoprecipitates from control and EGF-stimulated cells expressing wild-type and 1028-EGFR was analyzed by filter binding with 14-3-3ζ. As seen in Fig. 3, complex formation between the truncated receptor and 14-3-3ζ was not detected. Our results suggest that the carboxy-terminal domain of the EGFR is involved in the interaction with 14-3-3ζ, either directly, or indirectly involving another unknown kinase. When searching for potential interaction sites in this receptor domain, we found a

Table 1
Binding preferences of 14-3-3 and proposed 14-3-3 binding motif in the processed EGFR

14-3-3 binding preferences: RSX(pS/T)XP or RXXX(pS/T)XP	
Sequence	Protein
(1040)SFLQ**R**Y**S**SD**P**TGAL	EGFR human
(1040)AFLQ**R**Y**S**SD**P**TGAV	EGFR mouse
(1086)SPLQ**R**Y**S**ED**P**TVPL	ErbB2 human
(1079)KDGN**R**L**S**QP**P**NCSQ	Let-23 C-elegans

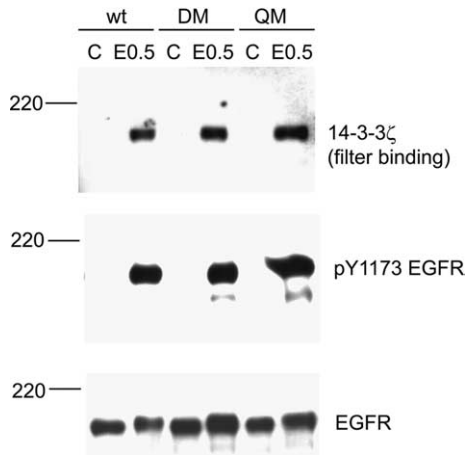

Fig. 4. Analysis of 14-3-3ζ binding to EGFR serine mutants. Total lysates from NIH3T3 cells expressing wild-type EGFR (wt), an EGFR serine double mutant (DM; with serine residues 1046 and 1047 removed) and an EGFR serine quadruple mutant (QM; with serine residues 1046, 1047, 1057 and 1142 removed) were examined. Lysates from untreated cells (C) or cells stimulated with EGF for 0.5 min were separated by SDS–PAGE and transferred to nitrocellulose filter. The filter was analyzed by immunoblotting with a rabbit anti-14-3-3 antibody (top panel), a rabbit anti-pY1173 antibody (middle panel), and a sheep anti-EGFR antibody (bottom panel). The markers at left indicate 220 kDa.

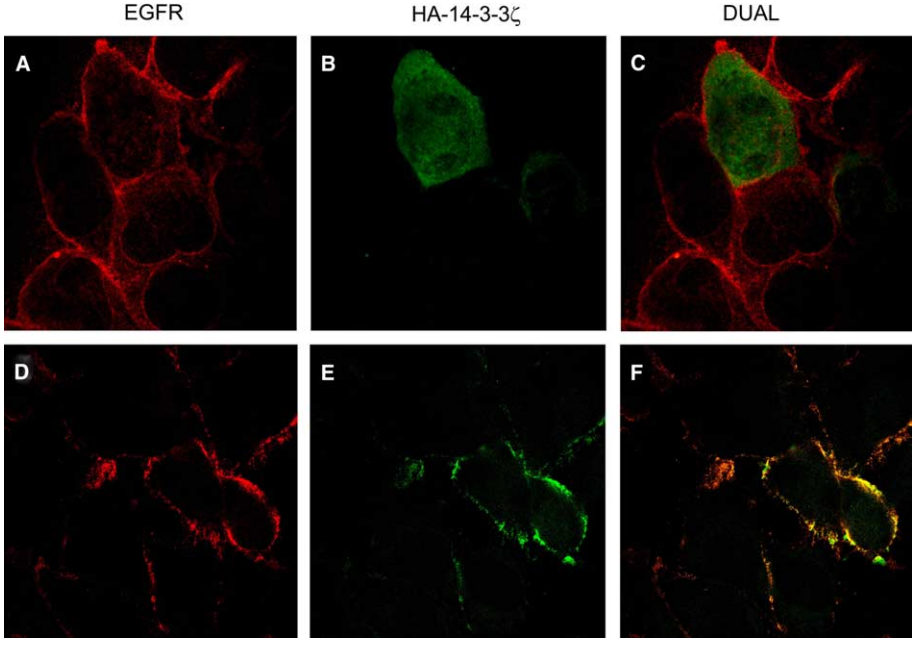

Fig. 5. Immunolocalization of EGFR and 14-3-3ζ. HeLa cells were transfected with a HA-tagged 14-3-3ζ and left untreated (A–C) or stimulated with 10 nM EGF for 1 min (D–F). A sheep anti-EGFR followed by Cy3-conjugated donkey anti-sheep antibody (A and D; green) and a mouse anti-HA followed by Cy2-conjugated donkey anti-mouse (B and E; red) were applied for detection. Bar, 10 μm.

serine-containing sequence that resembles the known binding preferences for 14-3-3 [10,18]. The 14-3-3 binding preference sequences and the proposed 14-3-3 binding sequence in human EGFR and additional species are presented in Table 1. To test the relevance of serine residues 1046 and 1047 (S1046, S1047) in 14-3-3 interaction, S1046 and S1047 were altered by site-directed mutagenesis to alanine. Analysis of EGFR immunoprecipitates from NIH3T3 cells stably expressing the wild-type EGFR and the S1046A/S1047A mutant by filter binding with 14-3-3ζ revealed that the mutant receptor did not exhibit a different 14-3-3 binding pattern in comparison to the wild-type EGFR (Double Mutant in Fig. 4). Two additional serine residues in the carboxy-terminal tail of the receptor were also mutated (S1057A and S1142A) without any effects on 14-3-3 binding to the EGFR (QM in Fig. 4). Although these experiments do not locate the exact binding site for 14-3-3 in the EGFR, they provide further proof that 14-3-3 proteins can bind to a variety of proteins without known 14-3-3 binding preference motifs [19–21] as well as non-phosphorylated proteins [22,23].

We utilized immunofluorescence microscopy to study the EGF-induced re-distribution of 14-3-3ζ and possible interaction with the EGFR. Cells were transfected with an HA-tagged 14-3-3ζ using Lipofectamine (Invitrogen) 24 h prior to experiments. In unstimulated cells 14-3-3ζ was located throughout the cytosol, whereas EGFR was found mainly in the plasma membrane (Fig. 5A–C). After stimulation with EGF for 1 min, we observed a redistribution of 14-3-3ζ to the plasma membrane where it was co-localized with the EGFR (Fig. 5D–F).

In summary, our results show that 14-3-3ζ can interact with the EGFR through an activation-dependent mechanism. We also determined that the 14-3-3ζ-EGFR association involves the carboxy-terminal receptor domain, but the exact interaction site remains to be identified.

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