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Research article

Binding of alpha-ACTN4 to EGF receptor enables its rapid phosphorylation

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ABSTRACT

Alpha-ACTN4, a member of alpha-actinin family is critical for cell motility through its regulated binding of actin filaments. We previously found that EGF exposure of cells triggers the tyrosyl-phosphorylation of ACTN4 in fibroblasts that dramatically downregulates its binding to actin filaments. However, the exact kinase remained uncertain. In the present study, we report that the phosphorylation of ACTN4 occurs within seconds upon EGF treatments and is accomplished via direct interaction of ACTN4 with the EGF receptor. The major binding domain of ACTN4 for EGF receptor is mapped to the N-terminal 32 amino acids. A second domain minimizes the interaction, as truncation of the C-terminal tail enhances ACTN4 binding to EGF receptor. A mimetic phosphorylated ACTN4, Y4/31E, presents low binding to EGF receptor. Overexpression of EGF receptor in melanoma cell lines, also accomplishes the phosphorylation of ACTN4 in the presence of EGF. These findings suggest that the binding of ACTN4 to EGFR enables its direct and rapid phosphorylation resulting in dissociation from EGFR and decreased binding to actin filaments.

1. Introduction

Alpha-actinin-4 (ACTN4) belongs to the alpha-actinin family originally known to crosslink actin filaments to maintain the stability of cytoskeleton and regulate cell motility [1, 2]. ACTN4 modulation is required for cell locomotion, as its affinity for actin filaments is regulated by signaling through growth factor receptors including the EGF receptor (EGFR) and the TAM family kinases [3, 4]. This is critical for cell movements during wound healing [5] and tumor invasion [6, 7].

The alpha-actinin family consists of four members. Among them, alpha-actinin-1 (ACTN1) and ACTN4 are ubiquitously expressed in non-muscle cells. Although ACTN1 and ACTN4 are highly homologous in amino acid sequence, ACTN4 has an extended N-terminus including an additional 19 amino acids. This unique region contains two differentiating targets, a tyrosine phosphorylation site (Y4) and a calpain cleavage site between tyrosine 13 and glycine 14 [8,9]. The first 45 N-terminal amino acids constitute an unstructured domain and are thus invisible in 3-D crystal structures that visualize the calponin homology 1 and 2 domains (CH1 & CH2) and three actin binding sites of ACTN4 [10,11]. This leaves the roles of this regulatory region unknown.

EGF-induced phosphorylation of ACTN4 at tyrosine 4 and 31 significantly diminishes its binding to actin filaments [8, 12]. This phosphorylation of ACTN4 presents resistance to the cleavage by m-calpain, a process that is proposed to lock ACTN4 bridging of the membrane to the actin cytoskeleton. This reduced actin binding also restores the mis-localization of K255E ACTN4, a mutation in human patient that causes focal segmental glomerulosclerosis [7, 13]. These findings suggest that phosphorylation regulates the function of ACTN4. However, which kinase directly phosphorylates ACTN4 when cells are stimulated with EGF remains unclear. In the present study, we show that EGFR rapidly and directly phosphorylates ACTN4 via a physical interaction. The binding site of ACTN4 for EGFR is mapped to its N-terminal unstructured domain of the first 45 amino acids.

2. Materials and methods

2.1. Cell culture and reagents

NR6WT fibroblasts, originally constructed by stably expressing human EGFR in murine 3T3 fibroblasts that were selected to lack endogenous EGFR [14, 15] were grown in alpha-MEM media supplemented with 7.5% fetal bovine serum, 1x non-essential amino acids, 1x sodium pyruvate, 1x L-glutamine and 1x pen/strep (100 units/ml penicillin; 100 μg/ml streptomycin) antibiotics. Melanoma cell line WM1158 and WM983b cells were cultured in DMEM (1 gL^{-1} glucose) : L-15 3:1 medium containing 10% fetal bovine serum and 1x pen/strip antibiotics.

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Plasmid transfection reagent xFect, Plasmid isolation kit and PCR extraction kit were purchased from Clontech/Takara Life Technologies (Grand Island, NY). Monoclonal GFP antibody conjugated to agarose beads (GFP-Trap A) was purchased from ChromoTek GmbH Planegg-Martinsried, Germany). Monoclonal EGFR antibody conjugated agarose (EGFR antibody (A-10) AC, normal mouse IgG-AC and monoclonal GFP were purchased from Santa Cruz (Dallas, TA). Monoclonal phospho-tyrosine antibody (p-Tyr-100) and polyclonal antibodies against phospho-EGFR (Y1173) and phospho-p38, phospho-ERK (Thr202/Tyr204) were purchased from Cell Signaling Technology (Beverly, MA). AccuPrime™ *Pfx* SuperMix was purchased from Invitrogen/Thermo Fisher Scientific (Carlsbad, CA). Restriction enzymes were purchased from New England Biolabs (Ipswitch, MA).

2.2. Mutagenesis and construction of expression vectors

Truncations of ACTN4 were accomplished by polymerase chain reaction (PCR) mutagenesis. All primers used in mutagenesis are:

32-911 sense 5′-cgcgaattacgatggcccaggaggacgactgggacc-3′
46-911 sense 5′-cgcgaattccgatgccggcctgggagaagcagcagcgcaagac-3′
ACTN4 full length antisense 5′-cgcggtacccgcaggtcgctctcgccatacaaggccg-3′
deletion of amino acids 46-270
sense 5′- cgggacctgctgctggacgcgcagaaggctgaaactgccgcc-3′
antisense 5′-cagccttctgcgcgtccagcagcaggtcccggtcccagtcg-3′.

PCR was performed using AccuPrime™ *Pfx* polymerase on a thermal cycler for 25 cycles of denaturing for 30 s at 95 °C, annealing for 30 s at 60 °C and extending for 3 min at 68 °C. PCR products were applied on 1% agarose gel for electrophoresis. Interested DNA band was excised from gel and then extracted using DNA extraction kit. Pure DNA fragment completely digested by restriction enzyme EcoR I and Kpn I followed by inactivation for 25 min at 75 °C was cloned into mammalian expression vector pEGFP-N1 (Clontech/Takara Life Technologies) digested with EcoR I and Kpn I to fuse an eGFP tag at the carboxyl tail of ACTN4. All positive clones were confirmed by restriction digestion and DNA sequencing analyses.

2.3. Transfections

Transfection of NR6WT fibroblasts and melanoma cells was performed according to the manufacture instructions as previous describing [13]. In brief, 300,000 cells were plated in each well of six-well tissue culture plate in growth medium to reach ~90% confluency next day. To form a complex of polymer and DNA for each well, 4μg of endotoxin-free plasmid was diluted into 100 μl xFect reaction buffer and mixed completely by pipetting up and down for 5 times. Then 1.5 μl of xFect polymer was added into the diluted DNA solution immediately followed by vortexing at highest speed for 10 s. The DNA-polymer mixture was incubated for 10 min at room temperature without disturbance. During incubation, the growth medium was changed to quiescence medium (same components as growth medium except the content of FBS) containing 0.1% dialyzed FBS. Lastly, the mixture was slowly added into the culture medium and the cells further incubated overnight prior to analyzing.

2.4. Immunoprecipitations

For immunoprecipitation, cells grown in six-well plate were rinsed with PBS (-Ca^{++}, -Mg^{++}) prior to adding 200 μl per well of ice-cold immunoprecipitation buffer (10 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% Triton X-100) containing 1x protease inhibitors cocktails set V from EMD Millipore (Billerica, MA). The cell lysate was collected from each well and combined (3 wells for NR6WT fibroblasts and 6 wells for melanoma cells for each sample) by scaping into a microcentrifugation tube for centrifuging for 30 min at 4 °C. Then, the supernatant was carefully transferred to a new microcentrifugation tube containing a mix of 5 μl of GFP-Trap A and 15 μl normal mouse IgG-AC agarose (for GFP immunoprecipitation) or 20 μl of EGFR antibody AC agarose (for EGFR immunoprecipitation) beads followed by gently shaking for 4h or overnight at 4 °C. Finally, beads were washed with immunoprecipitation buffer for 4 times and then eluted with 20 μl of 2x SDS protein sample buffer. Eluted protein was boiled for 3 min and subjected to electrophoresis and immunoblotting.

2.5. Immunoblotting

The interactions of proteins were analyses by examining all the immunoprecipitated GFP-tagged ACTN4 protein or 20 μg of total cellular proteins of each treatment; these were loaded on a concentration of SDS-polymerized acrylamide gel for optimum separation of the target protein. After electrophoresis, proteins were transferred to PVDF membrane from gel using 20% methanol containing transfer buffer for 1 h at a constant 350 mA current. Then the PVDF membrane was incubated with 5% fat free milk for 30 min at room temperature followed by incubation with 1:1000 diluted appropriate primary antibody overnight at 4 °C. The next day, the membrane was washed by TBS (Tris Buffered Saline) buffer containing 0.1% Tween-20 for 3 times, 10 min each prior to incubating with 1:2000 diluted horse radish peroxidase (HRP) labeled secondary antibodies. After one hour incubation at room temperature the membrane was completely washed and then developed on a film.

2.6. Isolation of cellular cytoskeleton

Isolation of cytoplasm and cytoskeleton was performed according to the manufacture instructions as previous describing [9]. In brief, cells were washed with cold PBS followed by incubation with 1x cellular extraction buffer on ice for 2 min. Then soluble cellular compartment was collected after pipetting up and down three times and labeled as “S”. The compartment still residing on Petri dish was carefully washed once with 1x cytoskeleton wash buffer before incubating with 1x nuclear extraction buffer on ice for 10 min. After aspirating the cytoskeleton remaining on Petri dish was gently washed twice with 1x cytoskeleton wash buffer and then was extracted with 1x cytoskeleton solubilization buffer containing 1x SDS sample buffer. The cytoskeleton extract was labeled as “C”. The proteins of both “S” and “C” were separated by SDS-PAG electrophoresis for immunoblottings against appropriate antibodies.

3. Results

3.1. EGF exposure leads to rapid phosphorylation of ACTN4

Besides bridging actin filaments to the membrane, ACTN4 has been suggested to interact with many other proteins [3, 16, 17]. Among those proteins, EGFR is of interest as we previously reported that EGF stimulation triggers the phosphorylation of ACTN4 in NR6WT fibroblasts. Therefore, we hypothesized that EGFR directly and rapidly phosphorylates ACTN4. To test this hypothesis, we used NR6WT fibroblasts to transiently express ACTN4 with an enhanced-GFP (eGFP) tag protein at its carboxyl terminus for specific and efficient immunoprecipitation of exo-ACTN4 and the detection of its phosphorylation at tyrosine. As shown in Figure 1A, we detected a significant phosphorylation of ACTN4-eGFP at tyrosine, upon exposure to EGF for only half minute. The level of ACTN4 phosphorylation increased with time of EGF stimulation and reached a plateau at 5 min. Both MAP kinase p38 and ERK were activated after EGF stimulation, as expected, but this was noted only after 2 min indicating the EGF-mediated phosphorylation of ACTN4 is not likely through the activation of p38 or ERK. As a control, the activation of EGFR could be seen at the half-minute time point.

As the phosphorylation of ACTN4 at the half-minute time point of EGF stimulation was significant, we treated cells for shorter time to

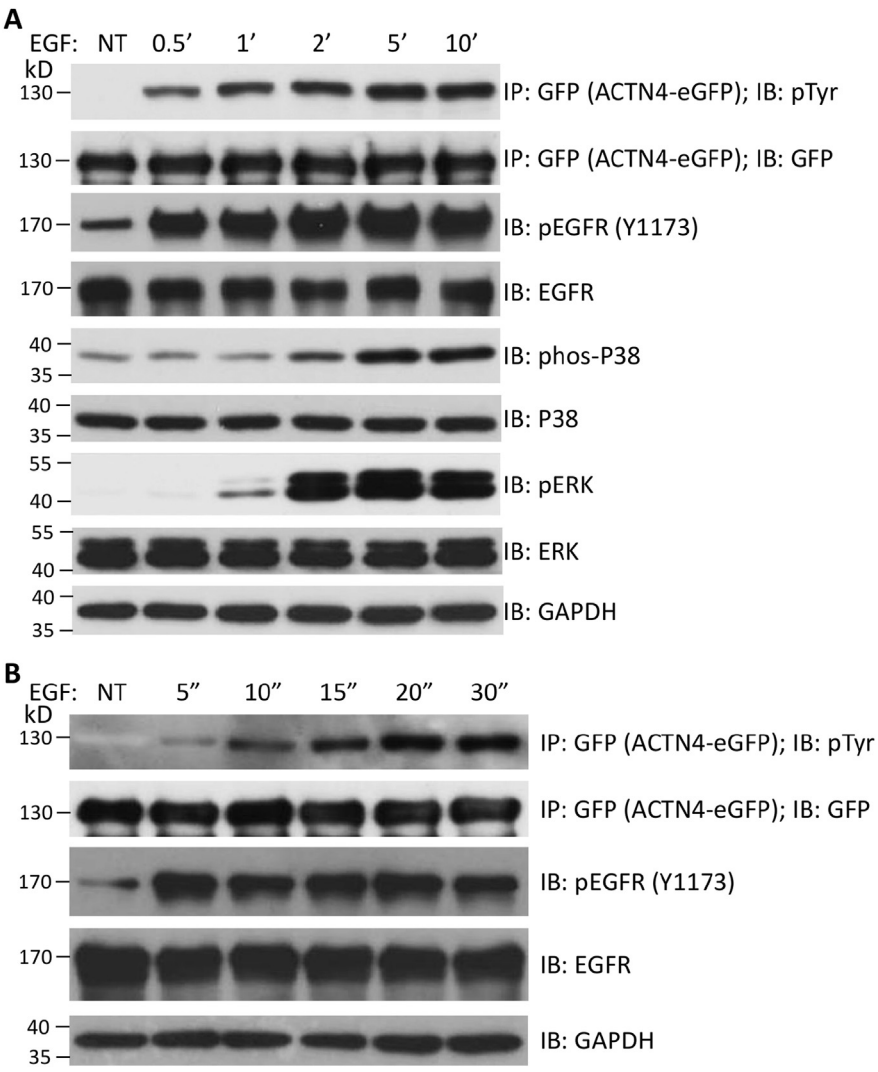

Figure 1. ACTN4 is rapid phosphorylated in RN6WT fibroblasts. (A, B) Immunoblottings of tyrosyl phosphorylation (1st panel) and total protein (2nd panel) of ACTN4-eGFP immunoprecipitated using antibody conjugated agarose beads directed against eGFP, from quiescent NR6WT fibroblasts stimulated with 10 nM EGF for indicated time. The other panels (A: 3rd to 9th; B: 3rd to 5th) are immunoblottings of total cellular lysate of NR6WT fibroblasts transiently transfected with ACTN4-eGFP and then treated with 10 nM EGF for indicated time using indicated antibodies. (A) Time course from 0.5 to 10 min. (B) Time course from 5 to 30 s. Shown are representative of three experiments.

determine how fast the ACTN4 phosphorylation can be triggered in NR6WT fibroblasts. To our surprise, ACTN4 was phosphorylated when cells were stimulated with EGF for only five seconds (Figure 1B). Taken together, these data suggest that EGFR itself directly phosphorylated ACTN4 in NR6WT fibroblasts, or was in a direct complex with ACTN4 prior to stimulation.

3.2. The N-terminus of ACTN4 is required for its binding to EGFR

The above finding and an earlier report [3] suggest that EGFR interacts with ACTN4. We also found co-immunoprecipitation using an EGFR antibody. As shown in Figure 2A, ACTN4 was co-immunoprecipitated with EGFR suggesting that EGFR interacts with ACTN4 in NR6WT fibroblasts. To determine whether this interaction may be due to non-specific binding of the eGFP tag, we overexpressed another membrane to cytoskeleton bridging protein, vinculin-eGFP, in these cells; the absence of vinculin-eGFP being retained on the EGFR antibody agarose beads excluded the possibility that the co-immunoprecipitation of ACTN4-eGFP with EGFR is due to the interaction of eGFP itself and EGFR. This specific interaction between EGFR and ACTN4 was also confirmed by the co-ordinate co-immunoprecipitation of endogenously expressed ACTN4 with the endogenous EGFR (Figure 2B and 3A). To further confirm this interaction, immunoprecipitation using an antibody against GFP in the ACTN4-eGFP construct also fished out EGFR in reverse (Figure 2C).

Similar to other alpha-actinin family members, ACTN4 consists of three major domains including an N-terminal actin-binding domain, central spectrin repeats domain, and C-terminal calcium binding domain. To map which domain is required for it's binding to EGFR, we created a series of ACTN4 mutants by truncation and determined their binding to EGFR. As shown in Figure 2D, compared to full length WT ACTN4, the fragment 1–841 lacking the calcium binding motif 2 bound more EGFR suggesting that the C-terminal is an inhibitory domain for its binding to EGFR. Fragment 1–271 containing the entire actin-binding region also presented higher binding activity indicating the binding domain for EGFR localizes within this fragment and further supporting that the C-terminus contains an inhibitory domain.

The phosphorylation sites of ACTN4 are tyrosine 4 and 31 [8]. We next truncated the first 31 amino acids (mutant 32–911) to remove both phosphorylation sites to see if the binding domain localizes within this region. As shown in Figure 2E, mutant 32–911 presented significantly decreased binding activity to EGFR. Further truncation such as 46–911 was performed to remove the whole unstructured domain. We found that the binding activity of mutant 46–911 was similar with mutant 32–911. Deletion of 32–45aa region only slightly decreased its binding to EGFR.

Interestingly, Y4/31E, a mutant of ACTN4 in which tyrosine (Y) 4 and 31 were replaced with glutamic acids (E) to mimic phosphorylated ACTN4 presented a dramatic decrease in its binding to EGFR (Figure 2D top panel lane 2 vs 1). This finding further suggests that

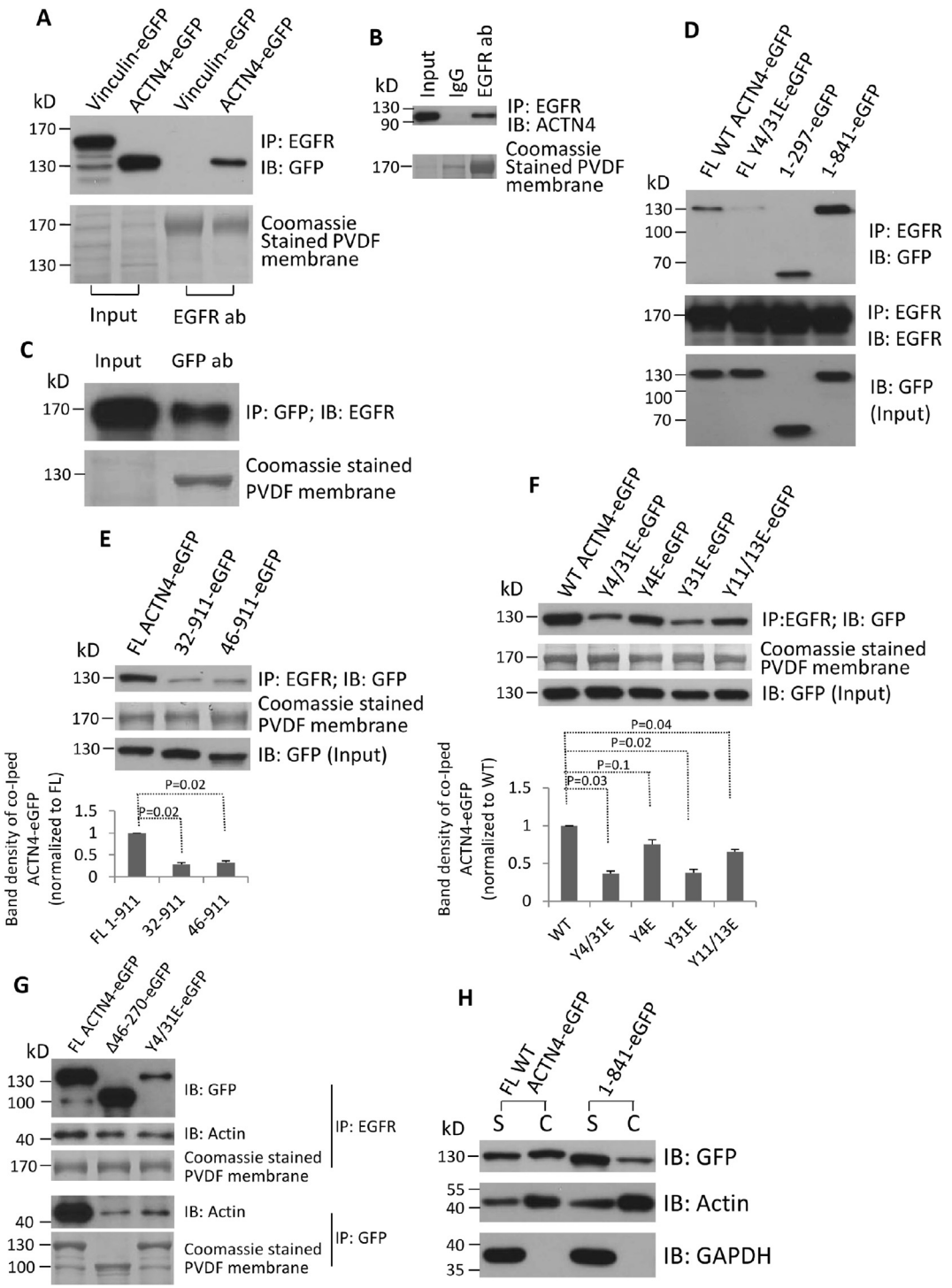

Figure 2. ACTN4 interacts with EGFR. (A) Immunoblotting of ACTN4-eGFP, or vinculin-eGFP as the negative control, co-immunoprecipitated using anti-EGFR antibody. Input stands for total cellular lysate (top panel). Coomassie stained PVDF membrane shows the immunoprecipitated EGFR protein. (B) Immunoblotting of endogenous NR6WT ACTN4 co-immunoprecipitated with EGFR using EGFR antibody (top panel). Immunoprecipitated EGFR protein on PVDF membrane was stained with Coomassie blue (bottom panel). Input stands for total cellular lysate. IgG stands for normal mouse IgG-agarose. (C) Immunoblotting of EGFR co-immunoprecipitated using anti-GFP antibody. Input stands for total cellular lysate (top panel). Coomassie stained PVDF membrane shows the immunoprecipitated ACTN4-eGFP protein. (D) Immunoblotting of full length WT and mutant ACTN4-eGFP co-immunoprecipitated using anti-EGFR antibody (top panel). Input stands for start material of immunoprecipitation immunoblotted with GFP antibody (middle panel). Immunoprecipitated EGFR is blotted with EGFR antibody (bottom panel). (E, F) Immunoblotting of full length WT and mutant ACTN4-eGFP co-immunoprecipitated using EGFR antibody (top panel). Input stands for start material of immunoprecipitation immunoblotted with GFP antibody (middle panel). Immunoprecipitated EGFR is stained with Coomassie (bottom panel). Graph stands for the band density of co-immunoprecipitated ACTN4-eGFP. Data are mean of ±SD of three independent experiments. Statistical analysis was performed using Student's t-test. (G) Immunoblotting of ACTN4-eGFP (1st panel) and actin (2nd panel) co-immunoprecipitated using EGFR antibody. Coomassie stained PVDF membrane (3rd panel) shows the immunoprecipitated EGFR protein. Immunoblotting of actin (4th panel) co-immunoprecipitated using GFP antibody. Coomassie stained PVDF membrane (5th panel) shows the immunoprecipitated GFP-tagged ACTN4 protein. (H) Immunoblottings of indicated proteins in isolated cellular plasma (S) and cytoskeleton (C). Shown are representative of three experiments.

the binding domain of ACTN4 for EGFR localizes within its N-terminal tail. Therefore, we were interested in wondering whether Y31 is the key residue in the regulation of the binding of ACTN4 to EGFR similar to its role in diminishing the actin binding activity of ACTN4 [6]. As shown in Figure 2F, Y4E presented slightly lower binding to EGFR compared to WT but like Y4/31E, the binding activity of Y31E was significantly diminished implying that the phosphorylation of Y31 plays an important role in the regulation of ACTN4 binding to EGFR. Furthermore, Y11/13E, the phosphor-mimetic of TAM family kinase phosphorylation of EGFR [4], showed only a moderate decrease in the binding to EGFR (Figure 2F). Taken together, these data suggest that the major binding domain of ACTN4 for EGFR reside within the flexible N-terminal region ranging from 1 to 32 amino acids.

As both EGFR and ACTN4 are actin-binding proteins [1, 18, 19], it is possible that ACTN4 binds to EGFR in a complex with actin as a bridge instead of a direct interaction between them. To exclude this possibility, we constructed a mutant ACTN4$_{\Delta 46\text{-}270}$ in which all three actin-binding sites were completely deleted. As shown in Figure 2G, co-immunoprecipitation of the ACTN4$_{\Delta 46\text{-}270}$ presented a negligible amount of actin compared to WT ACTN4 (4th panel lane 2 vs 1), but this mutant presented similar binding to EGFR as WT ACTN4 (1st panel lane 2 vs 1). This result suggests that actin does not bridge the interaction of EGFR and ACTN4. The content of co-immunoprecipitated Y4/31E was much weaker than WT even though the same trace amount of actin was present in the sample; this further supports the contention that the co-immunoprecipitation of ACTN4-eGFP with EGFR antibody is not due to the binding of eGFP itself or actin-bridging to EGFR. Furthermore, Figure 2D shows that ACTN4 truncated mutant 1–841 lacking the C-terminal 70 amino acids presents enhanced binding to the EGFR compared to full length WT ACTN4. Combining the dramatically impaired actin binding activity of mutant 1–841 (Figure 2H) and rapid phosphorylation of ACTN4, these results suggest that ACTN4 directly binds to EGFR.

3.3. EGF triggers ACTN4 phosphorylation in melanoma cells

Our previous findings showed a role for ACTN4 in regulating the morphology, motility and invasion of melanoma cells *in vitro* [7]. As melanomas present autocrine EGFR signaling, we were interested in determining whether the interaction between EGFR and ACTN4 also occurs in melanoma cells. Therefore, we transiently cotransfected melanoma cell lines WM983b and WM1158 cells with EGFR and ACTN4-eGFP. As shown in Figure 3B and 3D, ACTN4 was co-immunoprecipitated with EGFR antibody for both WM983b and WM1158 cells. Most importantly, EGF stimulation significantly triggered the phosphorylation of ACTN4 in these two cell lines (Figure 3C and 3E). These data further confirm that ACTN4 interacts with EGFR followed by rapid phosphorylation of ACTN4 when the cells are stimulated with EGF.

4. Discussion

Although ACTN1 and ACTN4 share high homology in nucleotide and amino acid sequences and both can cross-bridge actin stress fibers, they differ in their subcellular distributions and functions [1, 2]. ACTN4 is more widely distributed in cells being particularly evident in stress fibers and focal adhesions, underscoring its role in motility. Upregulated expression of ACTN4 is found in metastatic cancers, and thus is considered as a biomarker for the diagnosis of cancer progression [20, 21, 22, 23]. Our present study and that of other groups show that ACTN4 localizes on or near the plasma membrane [24, 25] and that this may involve direct interactions with transmembrane receptors that trigger motility. The physical interaction of ACTN4 and EGFR enables a direct and rapid phosphorylation of ACTN4 in fibroblasts. ACTN4 tyrosyl-phosphorylation increased after exposure to EGF and reached a plateau within 5min. The reason why the phosphorylation level of ACTN4 did not continue under extended EGF stimulation was due to the dephosphorylation mediated by phosphatase as our previously finding showed that a

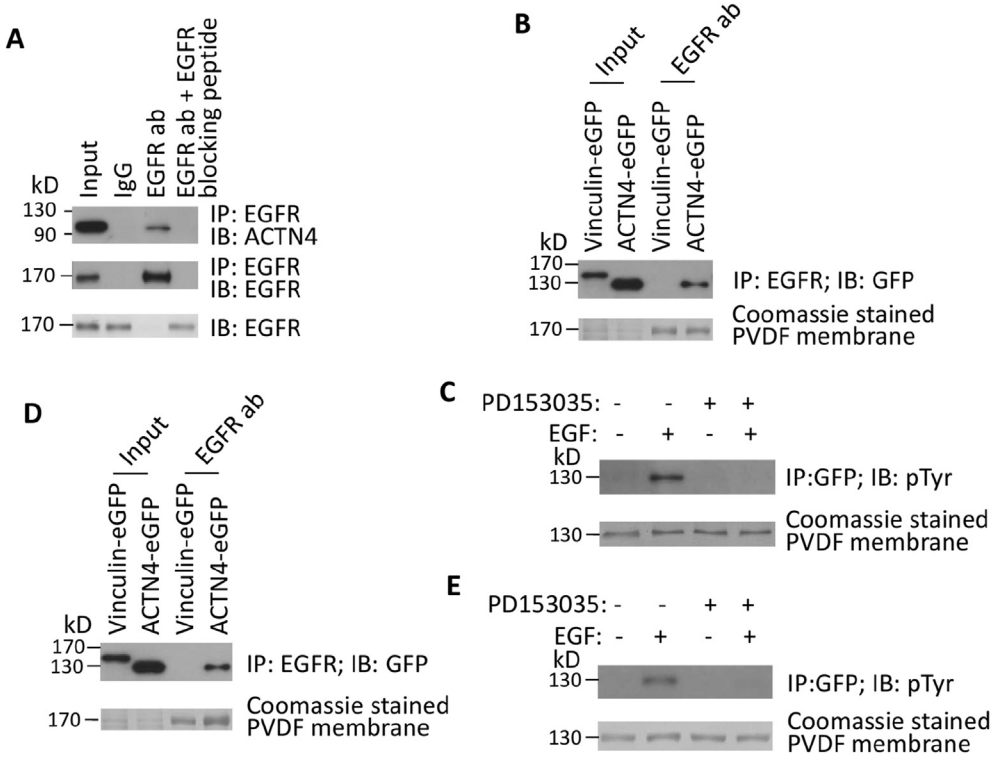

Figure 3. ACTN4 is phosphorylated in melanoma cells. (A) Immunoblotting of endogenous WM1158 ACTN4 co-immunoprecipitated with EGFR using EGFR antibody (top panel). Immunoblotting of immunoprecipitated EGFR protein (middle panel). Immunoblotting of EGFR protein in the flow through of immunoprecipitation (Bottom panel). Input stands for total cellular lysate. IgG stands for normal mouse IgG-agarose. (B, D) Immunoblotting of ACTN4-eGFP immunoprecipitated using anti-EGFR antibody (top panel). Immunoprecipitated EGFR transferred to PVDF membrane were stained with Coomassie after immunoblotting (bottom panel). Input stands for total cellular lysate. (C, E) Immunoblotting of phosphorylated ACTN4-eGFP at tyrosine 4 and 31 of ACTN4 (top panel). Protein ACTN4-eGFP transferred to PVDF membrane was stained with Coomassie blue (bottom panel). Melanoma cell line WM983b (C) and WM1158 (E) were transiently transfected with pcDNA3.1(-)-EGFR to express EGFR and pEGFP-N1-ACTN4 to express ACTN4-eGFP fusion protein and then pretreated with EGFR inhibitor PD153035 for 30 min prior to EGF stimulating for 10 min. Cellular lysate was subjected for immunoprecipitation using GFP antibody. Shown are representative of three experiments.

pretreatment of sodium vanadate, a phosphatase inhibitor significantly enhanced the ACTN4 phosphorylation [8]. These data suggest that the submembrane localization of ACTN4 may be due to interactions with transmembrane receptors, but at a minimum this localization affords direct modulation by mitogenic receptors.

In addition to bundling actin filaments, ACTN4 is also involved in the mediation of signaling transduction [2, 26]. Accumulating evidence indicates that ACTN4 itself is regulated at different levels including phosphorylation, m-calpain cleavage, calcium modulation and lipid binding [8, 9, 10, 27]. EGF-mediated phosphorylation of ACTN4 decreases its actin binding activity and its resistance to m-calpain cleavage [8, 9]. Consistent with this, the present study found that phosphor-mimicry at Y4/31 (Y4/31E) binds EGFR to a lesser extent than WT ACTN4 (Figures 2D, 2F and 2G). These findings are supported by the designation of the interactive region mapping to the N-terminal 32 amino acids of ACTN4. This suggests a mechanism by which ACTN4 leaves the EGFR once is it phosphorylated and releases actin filaments; this would allow for regulation of ACTN4 functioning for motility through a tissue that would not be available if the ACTN4 did not dissociate from an activated EGFR.

To date, the crystal structure of the intact ACTN4 is still not resolved due to its large molecular weight. Although the actin binding domains of alpha-actinins, including CH1 and CH2, have been crystalized [28], the 3-D structure of the N-terminal beginning 46 amino acids is unavailable as this region is inherently unstructured and cannot be crystalized. Thus, we cannot map the structural alterations of the first 32 amino acids for the binding to EGFR or the alterations that release from the receptor upon tyrosyl-phosphorylation. However, phosphomimetic Y4/31E and Y11/13E presenting decreased binding to EGFR further suggest that the region of 1–32 amino acids is the major binding domain of ACTN4 for EGFR.

Although we were interested in examining whether abolishing the binding activity of ACTN4 for EGFR will make it unavailable for EGF-mediated phosphorylation at tyrosines, we could not perform this kind of assay as the binding domain of ACTN4 for EGFR localizes within the region of 1–32 amino acids which contains the phosphorylation sites tyrosine 4 and 31 for EGF stimulation. If we truncate the fragment of 1–32 amino acids, we will lose phosphorylation sites of ACTN4 resulting in no phosphorylation. Further, we have reported that many amino acid substitutions in the first 32 amino acids will result in a 'closed' and unavailable unstructured domain [6], confounding interpretation of lack of interactions. It makes sense for us to suggest the binding domain of ACTN4 for EGFR to 1 to 32 amino acids because this region contains phosphorylation sties for EGF stimulation and thus the interaction between ACTN4 and EGFR minimizes the distance of EGFR kinase and phosphorylation sites of ACTN4. This closest contact enables fast phosphorylation such as within seconds after EGF stimulation. In the absence of direct experimental evidence that shows a direct interaction between ACTN4 and EGFR, rapid phosphorylation of ACTN4 can suggest that ACTN4 directly interacts with EGFR. If there is a bridge between ACTN4 and EGFR or there is another EGFR downstream kinase, which phosphorylates ACTN4, the phosphorylation of ACTN4 could not occur within seconds. For example, activations of ERK and p38 became detectable 2 min after stimulation by EGF (Figure 1A). Furthermore, our findings also suggest that EGFR and ACTN4 form a precomplex prior to the addition of EGF ligand (Figures 2A, 2B and 2C). Y4/31E presenting low binding to EGFR implies a dissociation of ACTN4-EGFR complex once ACTN4 is phosphorylated upon EGF stimulation.

Overexpression of EGFR in melanoma cells increases the phosphorylation of ACTN4 in the stimulation of EGF implying that ACTN4 may be also phosphorylated in other cancer cells with enhanced EGFR level. As Y4/31E presents decreased binding to EGFR, autocrine activation of EGFR might cause a release of cytoskeleton or focal adhesion from cell plasma membrane through the phosphorylation of ACTN4. Therefore, ACTN4 may be considered as one of the therapeutic targets in cancers that present elevated levels of EGFR.

Declarations

Author contribution statement

Alan Wells: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hanshuang Shao: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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