Mutation of an N-terminal acidic-rich region of p115-RhoGEF dissociates α_{13} binding and α_{13}-promoted plasma membrane recruitment

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Abstract The Ras homology (Rho) guanine nucleotide exchange factor p115-RhoGEF couples the α_{13} heterotrimeric guanine nucleotide binding protein (G protein) subunit to Rho GTPase. α_{13} binds to a regulator of G protein signaling (RGS) domain in p115-RhoGEF, but the mechanism of α_{13} activation of p115-RhoGEF is poorly understood. In this report, we demonstrate in cell-based assays that the acidic-rich N-terminus, adjacent to the RGS domain, is required for binding to activated α_{13}, and refine the importance of this region by showing that mutation of glutamic acids 27 and 29 in full-length p115-RhoGEF is sufficient to prevent interaction with activated α_{13}. However, α_{13}-interacting deficient N-terminal mutants of p115-RhoGEF retain α_{13}-dependent plasma membrane recruitment. Overall, these findings demonstrate a critical role for the N-terminal extension of p115-RhoGEF in mediating binding to α_{13} and dissociate two activities of p115-RhoGEF: binding to activated α_{13} and translocation to the PM in response to activated α_{13}.

Key words: Heterotrimeric guanine nucleotide binding protein; Ras homology GTPase; Guanine nucleotide exchange factor; Membrane translocation

1. Introduction

Heterotrimeric guanine nucleotide binding proteins (G proteins) ($\alpha\beta\gamma$) function as molecular switches to couple activated heptahelical G protein-coupled receptors (GPCRs) to a variety of intracellular effectors. The heterotrimeric G protein α subunits are typically divided into four families, α_s, α_i, α_q, and α_{12}, based on sequence and functional similarities. α_{12} and α_{13}, which comprise the α_{12} family and share 65% amino acid identity, have been implicated in the regulation of cell growth, oncogenesis, cell morphological changes, and actin cytoskeleton rearrangements [1]. Some of these activities, particularly actin cytoskeleton rearrangements, are mediated by the Ras homology (Rho) family of small GTPases [2]. Rho is activated by members of a large family of proteins, termed Rho guanine nucleotide exchange factors (RhoGEF) [3], and one RhoGEF, called p115-RhoGEF, has been demonstrated to provide a direct link between α_{13} and Rho [4,5].

Like virtually all RhoGEFs, p115-RhoGEF contains a tandem Dbl homology/pleckstrin homology (DH/PH) domain. The DH domain catalyzes the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on Rho, while the PH domain is necessary for both DH domain activity [6] and regulated plasma membrane (PM) localization [7]. Using reconstitution of purified components, it has been clearly demonstrated that activated forms of α_{13} can directly stimulate the Rho exchange activity of p115-RhoGEF [4]. Moreover, inside cells, activated α_{13} or GPCRs that activate α_{13} can recruit p115-RhoGEF from the cytoplasm to the PM [6–8].

The connection between α_{13} and p115-RhoGEF is mediated, at least in part, by an N-terminal regulator of G protein signaling (RGS) domain found in p115-RhoGEF that provides a binding site for α_{13} [4,9–11]. More than 20 RGS proteins have been identified, and they share an approximately 120 amino acid conserved domain [12]. The RGS domain of p115-RhoGEF is required for α_{13}-mediated activation of its RhoGEF activity and for α_{13}-dependent PM recruitment [7,10,11]. In addition, most RGS proteins function to accelerate the intrinsic GTP hydrolysis activity of select G protein α subunits, and, indeed, the RGS domain of p115-RhoGEF is a GTPase activating protein (GAP) for α_{12} and α_{13} [5]. Thus, p115-RhoGEF is both an effector and negative regulator of α_{13}. Two additional RhoGEFs, PDZ-RhoGEF and LARG, also contain an RGS domain and may also be involved in coupling $\alpha_{12/13}$ to Rho [9,13–16]. The RGS domains of P115-RhoGEF, PDZ-RhoGEF and LARG show very weak identity to other RGS boxes, and crystal structures of RGS domains from p115-RhoGEF and PDZ-RhoGEF show that these RGS domains require an additional $\sim$60 C-terminal amino acids to form the structural RGS domain [17,18]. For these reasons, the RGS domains from the RhoGEFs have been termed RGSL, for RGS-like, or rgRGS, for RhoGEF RGS, domains.

In p115-RhoGEF, the core RGS domain consists of amino acids 44–233, as defined by structural analysis [17]. However, a recent study demonstrated that some portion of the N-terminal 42 amino acids is also critical for RGS domain function. Whereas an rgRGS domain consisting of amino acids 1–252 of p115-RhoGEF functions as a GAP for α_{13}, a 42–252 rgRGS domain loses its GAP activity although it partially retains the ability to bind activated α_{13} [10]. The experiments

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Abbreviations: G protein, guanine nucleotide binding protein; RGS, regulator of G protein signaling; Rho, Ras homology; GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; GAP, GTPase activating protein; GPCR, G protein-coupled receptor

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described in this report were designed to test whether the N-terminal extension of p115-RhoGEF's rgRGS domain affects the ability of full-length p115-RhoGEF to interact with α_{13} in cell lysates or intact cells. We demonstrate that deletion of p115-RhoGEF amino acids 1–31 abolishes the ability of p115-RhoGEF to co-immunoprecipitate with activated α_{13}. Furthermore, we show that mutation of glutamic acids 27 and 29 also blocks co-immunoprecipitation of p115-RhoGEF and α_{13}. Surprisingly, however, the N-terminal ~40 amino acid extension is not required for strong α_{13}-induced PM recruitment of p115-RhoGEF.

2. Materials and methods

2.1. Plasmids

The N-terminal Myc epitope (MEQKLISEED)-tagged p115-RhoGEF in pcDNA3 has been described [7,8]. The HA epitope-tagged (DVPDYA) pcDNA3HA Gα_{13}wt and pcDNA3HA Gα_{13}QL, containing the GTPase inhibiting Q226L mutation, were provided by J.S. Gutkind [14]. For generation of N-terminal deletions of p115-RhoGEF, Myc epitope-tagged fragments of p115-RhoGEF were amplified with forward and reverse primers containing a 5′ *Hin*dIII site and a 3′ *Xba*I site for subcloning into pcDNA3. The Stratagene QuikChange site-directed mutagenesis kit was used to replace the glutamates at positions 27 and 29, 34 and 36, and 40 and 41 with alanines to generate pcDNA3-Myc-p115(E27,29A), pcDNA3-Myc-p115(E34,36A) and pcDNA3-Myc-p115(E40,41A), respectively. The forward and reverse primers used were 5′-ggggctgcggatgcggat-3′ and 5′-atccgcatccgcagcccc-3′ for p115(E27,29A); 5′-gattttgcgaacgcgctg-3′ and 5′-cagcgcgttcgcaaaatc-3′ for P115(E34,36A); and 5′-aactcagcagcgcaaaac-3′ and 5′-gttttgcgctgctgagtt-3′ for p115(E40,41A). The correct DNA sequence of the mutants was confirmed by DNA sequencing of the entire open reading frame (Kimmel Cancer Center Nucleic Acid Facility).

2.2. Cell culture and transfection

HEK293 and COS cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and penicillin and streptomycin. Cells were seeded in 6 cm or six-well plates 24 h prior to transfection. Transient transfections were performed with FuGene 6 (Roche Molecular Biochemicals) according to the manufacturer's protocol.

2.3. Antibodies

The anti-HA mouse monoclonal antibody 12CA5 was from Roche Molecular Biochemicals, and the anti-Myc mouse monoclonal antibody 9E10 was from Covance. An anti-HA rabbit polyclonal antibody was from Santa Cruz Biotechnology. Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Alexa 488 and Alexa 594, respectively, were from Molecular Probes.

2.4. Immunoprecipitations and immunoblotting

Co-immunoprecipitation analyses were performed as described [7,8]. Briefly, 72 h after transfection, COS cells were lysed in 500 μl lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 mM β-glycerophosphate, and 1 mM sodium vanadate). After incubation on ice for 1 h, cell suspensions were centrifuged at 1500×*g* for 5 min at 4°C. For AlF_4^- experiments, the supernatants were collected and divided into two aliquots. One aliquot was treated with 20 μM $AlCl_3$ and 20 mM NaF (+AlF_4^-) and the other part was untreated (−AlF_4^-). 9E10 or 12CA5 antibodies were added, and incubations were carried out for 1 h at 4°C. The immunocomplexes were recovered with the aid of Protein A/G agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Proteins in cell lysates and the immunocomplexes were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by immunoblotting.

2.5. Subcellular fractionation

Subcellular fractionation was performed as described previously [7,8]. 48 h after transfection, HEK293 cells were lysed in hypotonic buffer and separated into membrane-rich particulate (P) and cytoplasmic soluble (S) fractions by centrifuging the lysates at 100 000×*g* for 20 min as described. Equal volumes (i.e. equivalent cell fractions) of particulate (P) and soluble (S) fractions were loaded on SDS–PAGE for immunoblotting.

2.6. Immunofluorescence microscopy

HEK293 cells were grown on coverslips placed in six-well plates and were transfected with appropriate plasmids as described in the figure legends. 24 h after transfection, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized by incubation in blocking buffer (2.5% non-fat milk and 1% Triton X-100 in triethanolamine-buffered saline (TBS)) for 30 min. Cells were then incubated with 5 μg/ml anti-myc mouse monoclonal antibody 9E10 and a 1:100 dilution of anti-HA rabbit polyclonal antibody in blocking buffer for 1 h. The cells were washed with blocking buffer and incubated with a 1:100 dilution of goat anti-mouse Alexa 488 antibodies and goat anti-rabbit Alexa 594 secondary antibodies for 30 min. The coverslips were washed with 1% Triton X-100/TBS, rinsed in distilled water, and mounted on glass slides with 10 μl of Prolong Antifade reagent (Molecular Probes, Inc.). Images were viewed with an Olympus BX60 microscope equipped with a 60×/NA1.4 objective. Images were recorded with a Sony DKC-5000 digital camera and transferred to Adobe Photoshop for digital processing.

3. Results

3.1. N-terminal deletion mutants of p115-RhoGEF are defective in co-immunoprecipitation with AlF_4^--activated α_{13}

To examine a role for the N-terminal extension (Fig. 1) in binding of full-length p115-RhoGEF to activated α_{13}, we generated a series of short deletions at the N-terminus, (22-912)p115, (32-912)p115, and (42-912)p115, in which 21, 31, and 41 amino acids, respectively, were removed. We first tested the ability of the N-terminal deletion mutants of p115-RhoGEF to interact with α_{13}, as measured by AlF_4^--dependent co-immunoprecipitation [7,8,10]. Co-immunoprecipitations were performed in a reciprocal manner. Immunoprecipitation with anti-Myc antibody tested the ability of Myc-tagged p115-RhoGEF or the various deletion mutants to 'pull down' α_{13} (Fig. 2A), while immunoprecipitation using anti-HA antibody tested the ability of HA-tagged α_{13} to 'pull down' p115-RhoGEF or its mutants (Fig. 2B). As shown previously [7,8,10], full-length p115-RhoGEF and α_{13} co-immunoprecipitated from lysates treated with AlF_4^- (Fig. 2A and B, lanes 1 and 5), but little or no co-immunoprecipitation was observed in the absence of AlF_4^- activation of α_{13} (Fig. 2A and B, lanes 1 and 5). With the N-terminal deletion mutants, only (22-912)p115 (Fig. 2A and B, lane 2), but not (32-

Fig. 1. p115-RhoGEF schematic and N-terminal amino acid sequence. A: A representation of the domain structure of p115-RhoGEF is presented. Amino acid numbering and the location of critical domains are indicated. The rgRGS domain is defined by structural analysis [17] while the DH and PH domains are defined by sequence comparisons [22] and functional analysis [6]. B: Sequence of amino acids 1–42 is indicated. Glutamic acids mutated to alanines in this study are underlined.

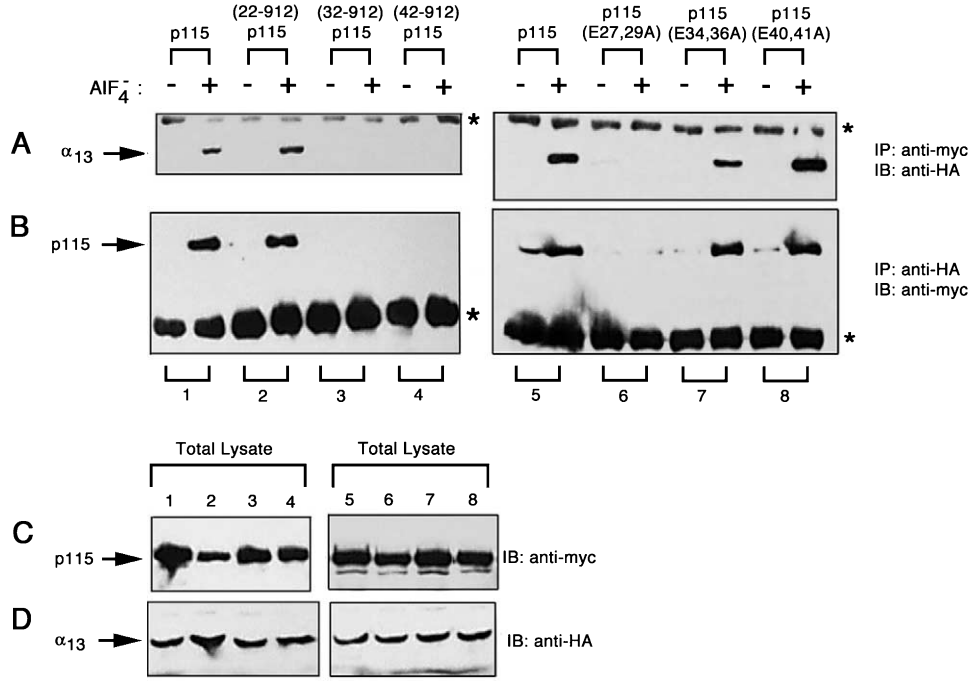

Fig. 2. Co-immunoprecipitation of AlF_4^--activated α_{13} and N-terminal mutants of p115-RhoGEF. COS cells were transfected with plasmids encoding HA-tagged wild type α_{13} (2 μg) along with Myc-tagged p115-RhoGEF (p115) or the indicated N-terminal mutants of p115-RhoGEF (0.5 μg). Cell lysates were either not treated (−) or treated (+) with AlF_4^-, as described in Section 2. A: Myc epitope-tagged proteins were immunoprecipitated using an anti-Myc monoclonal antibody and the immunocomplexes were immunoblotted using an anti-HA monoclonal antibody. B: In a separate set of transfections, identical to A, HA epitope-tagged wild type α_{13} was immunoprecipitated using anti-HA monoclonal antibody and the complexes were immunoblotted using an anti-Myc monoclonal antibody. The bands corresponding to the asterisk (*) represent precipitated immunoglobulin. C: The cell lysates from each transfection were immunoblotted using an anti-Myc monoclonal antibody to determine expression levels of p115-RhoGEF (lanes 1 and 5), (22-912)p115 (lane 2), (32-912)p115 (lane 3), (42-912)p115 (lane 4), p115(E27,29A) (lane 6), p115(E34,36A) (lane 7), and p115(E40,41A) (lane 8). D: The same blots from C were re-probed with an anti-HA monoclonal antibody to detect expression levels of α_{13} (lanes 1–8). The experiments in A–D are representatives of three similar independent experiments.

912)p115 or (42-912)p115 (Fig. 2A and B, lanes 3 and 4), co-immunoprecipitated with α_{13} from lysates treated with (+) AlF_4^-, suggesting that the 10 amino acids, 22–31, preceding the rgRGS domain (Fig. 1) are required for α_{13} binding.

3.2. *Glutamic acids 27 and 29 of p115-RhoGEF are essential for binding to activated α_{13}*

The short N-terminal extension prior to the rgRGS core domain contains an acidic stretch at amino acids 27–42 (Fig. 1). To further test the role of these acidic residues at the N-terminus, we generated mutants of p115-RhoGEF, where glutamic acids at positions 27 and 29, 34 and 36, and 40 and 41, were mutated dually with alanine residues, to generate p115(E27,29A), p115(E34,36A) and p115(E40,41A), respectively (Fig. 1). We observed virtually no co-immunoprecipitation of p115(E27,29A) with α_{13} in the presence of AlF_4^- (Fig. 2A and B, lane 6). On the other hand, marked AlF_4^--dependent co-immunoprecipitation of p115(E34,36A) and p115(E40,41A) with α_{13} was detected, similar to full-length p115-RhoGEF (Fig. 2A and B, lanes 7 and 8). These experiments refine the initial N-terminal deletions and indicate that one or both of glutamic acids 27 and 29 are critical for p115-RhoGEF to interact with α_{13}. Although the acidic-rich region extends beyond glutamic acids 27 and 29, mutation of glutamic acids 34, 36, 40 and 41 had no effect on the ability of p115-RhoGEF to interact with activated α_{13}.

3.3. *N-terminal mutants of p115-RhoGEF retain strong α_{13}-promoted PM recruitment*

We next examined the ability of a constitutively active mutant of α_{13}, α_{13}QL, to induce cytoplasm to PM redistribution of the N-terminal mutants of p115-RhoGEF. As we demonstrated previously [8], full-length p115-RhoGEF is found distributed throughout the cytoplasm (Fig. 3A, a), but, when α_{13}QL is co-expressed, p115-RhoGEF strongly localizes to the PM (Fig. 3A, b). Moreover, we demonstrated recently [7] that deletion of amino acids 1–245, which includes the rgRGS core domain and the N-terminal extension of p115-RhoGEF, completely prevented co-immunoprecipitation with activated α_{13} and abolished α_{13}-promoted recruitment to the PM (Fig. 3A, c and d). Thus, results with (246-912)p115 [7] suggested a correlation between loss of binding to α_{13} and loss of PM recruitment by α_{13}. In contrast, the α_{13} binding-defective N-terminal extension mutants showed a surprising retention of α_{13}-promoted PM recruitment. All the mutants were cytoplasmic when expressed alone (Fig. 3A, e and g; and not shown). When co-expressed with α_{13}QL, p115(E27,29A) (Fig. 3A, f) and (42-912)p115 (Fig. 3A, h) displayed strong PM localization, as did (22-912)p115, (32-912)p115, p115 (E34,36A) and p115(E40,41A) (not shown).

To extend the immunofluorescent microscopy results, subcellular fractionation into soluble (S) and membrane-rich particulate (P) fractions was performed. As shown previously [8], p115-RhoGEF was detected almost exclusively in the 'S' frac-

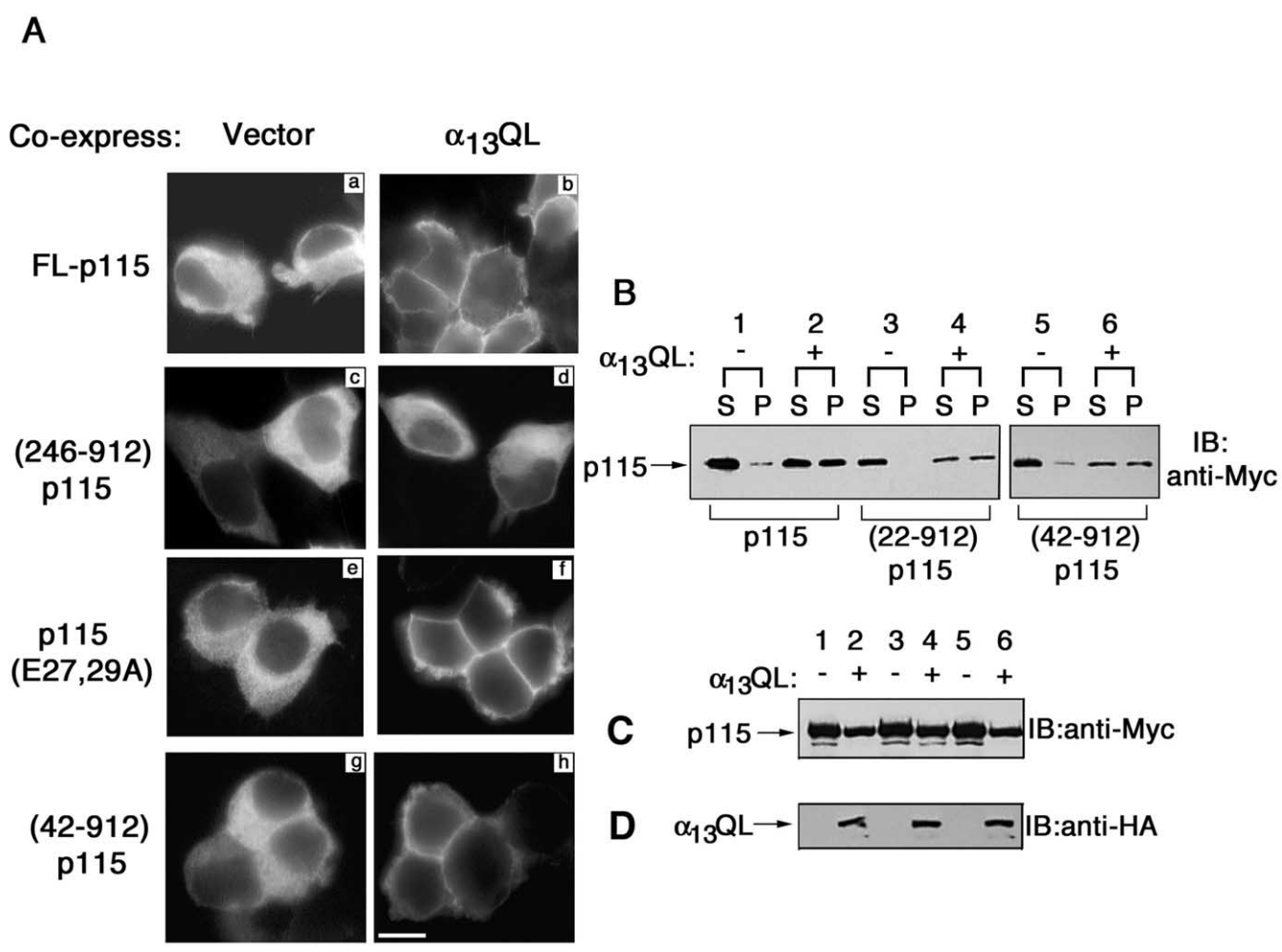

Fig. 3. Amino acids 1–41 are not required for α_{13}QL-mediated PM targeting of p115-RhoGEF. A: HEK293 cells were transfected with plasmids encoding full-length p115-RhoGEF (FL-p115) (a and b), (246-912)p115 (c and d), p115(E27,29A) (e and f) or (42-912)p115 (g and h) (0.05 μg), along with either empty pcDNA3 (a, c, e and g) or with a plasmid encoding α_{13}QL (b, d, f and h) (0.5 μg). Cells on coverslips were fixed and prepared for immunofluorescence microscopy as described in Section 2. Subcellular localization of p115-RhoGEF and N-terminal mutants was visualized by indirect immunofluorescence using an anti-Myc monoclonal antibody. Cells were also co-stained with an anti-HA polyclonal antibody to identify the cells expressing α_{13}QL (not shown). More than 100 cells were examined in at least three experiments and >95% cells showed staining patterns similar to the images presented. Bar, 10 μm. B: HEK293 cells in 6 cm plates were transfected with plasmids encoding full-length p115-RhoGEF (p115), (22-912)p115 or (42-912)p115 along with empty pcDNA3 (−) or pcDNA3 encoding α_{13}QL (+). After lysis in hypotonic buffer, cell lysates were separated into soluble (S) and particulate (P) fractions, as described in Section 2. The fractions were immunoblotted with an anti-Myc monoclonal antibody. C: Expression of p115-RhoGEF (lanes 1 and 2), (22-912)p115 (lanes 3 and 4) and (42-912)p115 (lanes 5 and 6) was determined by an immunoblot of cell lysates (from B) using an anti-Myc monoclonal antibody. D: Immunoblot in C was re-probed with an anti-HA monoclonal antibody to detect expression of α_{13}QL (lanes 2, 4 and 6). Experiments in B–D are representatives of three independent subcellular fractionation analyses.

tion when expressed with empty vector (Fig. 3B, lanes 1P and 1S) in HEK293 cells. However, a significant amount of p115-RhoGEF distributed into the 'P' fractions when expressed with α_{13}QL (Fig. 3B, lanes 2P and 2S), as expected [7,8]. Similarly, N-terminal extension mutants shifted from the 'S' to 'P' fraction upon α_{13}QL co-expression. As shown in Fig. 3B, (22-912)p115 and (42-912)p115 displayed substantial partitioning into the membrane-rich particulate 'P' fractions when co-expressed with α_{13}QL (Fig. 3B, lanes 3P and 3S, and lanes 4P and 4S). Taken together, the immunofluorescence microscopy (Fig. 3A) and cell fractionation (Fig. 3B) results indicate that the 41 amino acid N-terminal extension is not required for α_{13}QL-mediated PM translocation of p115-RhoGEF.

Deletion or mutation of residues in the N-terminal extension of p115-RhoGEF had no effect on Rho-dependent signaling (not shown), as measured by a serum response factor (SRF) transcriptional reporter assay. The N-terminal mutants and wild type full-length p115-RhoGEF displayed similar levels of basal activation of an SRF-luciferase reporter (not shown), indicating that N-terminal deletion or mutation did not disable the intrinsic Rho exchange activity. Unfortunately, we were unable to demonstrate whether or not activated α_{13} could effect an increase in activity of both wild type p115-RhoGEF and the N-terminal mutants of p115-RhoGEF inside cells, since we and others have been unable to demonstrate a synergistic activation of SRF transcription by combined expression of α_{13}QL and p115-RhoGEF [16,19]. Moreover, we have measured activation of RhoA directly using a well-described Rho pull-down assay [20], but, like the

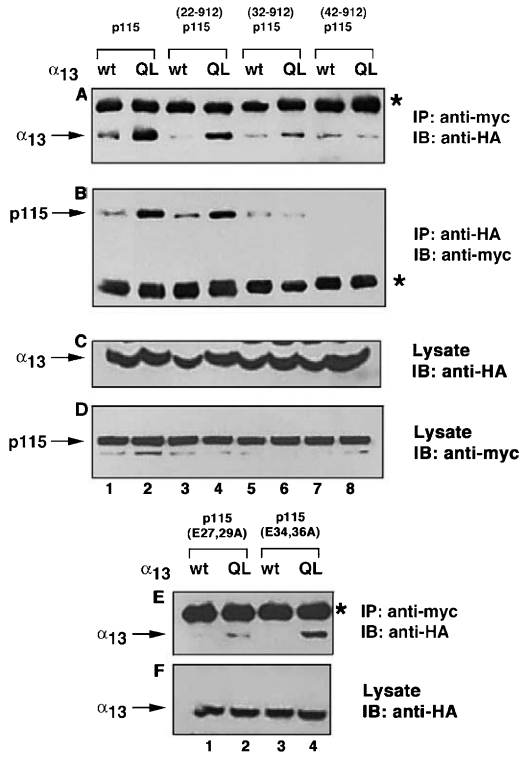

Fig. 4. Co-immunoprecipitation of constitutively active α_{13}QL and N-terminal mutants of p115-RhoGEF. COS cells were transfected with plasmids encoding full-length p115-RhoGEF (p115) or the indicated N-terminal deletion or point mutants (0.5 μg) along with plasmids encoding either wild type α_{13} or α_{13}QL (2 μg), as indicated. A: Myc epitope-tagged p115-RhoGEF and the N-terminal deletion mutants were immunoprecipitated from cell lysates using an anti-Myc monoclonal antibody. The immunoprecipitates were immunoblotted and probed with an anti-HA monoclonal antibody. B: In a separate experiment, COS cells were transfected in an identical manner as described above. HA-tagged wild type α_{13} or α_{13}QL were immunoprecipitated from cell lysates using anti-HA monoclonal antibody. The complexes were immunoblotted using anti-Myc monoclonal antibody. The bands corresponding to the asterisk (*) indicate precipitated heavy chain of immunoglobulin. C: The cell lysates used as the input for the immunoprecipitation in A were immunoblotted using an anti-HA monoclonal antibody to determine expression levels of α_{13} (lanes 1, 3, 5, and 7) or α_{13}QL (lanes 2, 4, 6, and 8). D: The cell lysates used as the input for the immunoprecipitation in B were immunoblotted using an anti-myc monoclonal antibody to determine expression levels of p115-RhoGEF (lanes 1 and 2), (22-912)p115 (lanes 3 and 4), (32-912)p115 (lanes 5 and 6), and (42-912)p115 (lanes 7 and 8). E: Myc epitope-tagged p115(E27,29A) (lanes 1 and 2) and p115(E34,36A) (lanes 3 and 4) were immunoprecipitated from cell lysates using an anti-Myc monoclonal antibody. The immunoprecipitates were immunoblotted and probed with an anti-HA monoclonal antibody to detect α_{13} (lanes 1 and 3) and α_{13}QL (lanes 2 and 4). F: The cell lysates used as the input for the immunoprecipitation in E were immunoblotted using an anti-HA monoclonal antibody to determine expression levels of α_{13} (lanes 1 and 3) and α_{13}QL (lanes 2 and 4).

SRF assay, we have not detected any synergistic activation of RhoA by combined expression of α_{13}QL and p115-RhoGEF (not shown).

3.4. *N-terminal mutants of p115-RhoGEF are defective in co-immunoprecipitation with constitutively active α_{13}QL*

An important difference between the co-immunoprecipitation assays (Fig. 2) and the subcellular localization assays (Fig. 3) is that AlF_4^--activated α_{13} was used in the former, while the constitutively active α_{13}QL mutant was used in the latter. α_{13}QL is a mutant lacking GTPase activity and therefore locked in a GTP-bound active form; however, AlF_4^--activated α_{13} is thought to mimic the transition state of GTP hydrolysis. Therefore, we tested the ability of α_{13}QL to interact, via co-immunoprecipitation, with p115-RhoGEF or the N-terminal deletion mutants of p115-RhoGEF. We detected co-immunoprecipitation of α_{13}QL with p115-RhoGEF and (22-912)p115 but not with (32-912)p115 or (42-912)p115, both in anti-Myc immunoprecipitates (Fig. 4A) or in anti-HA immunoprecipitates (Fig. 4B). Similarly, p115(E27,29A) displays little or no co-immunoprecipitation with α_{13}QL, while p115(E34,36A) interacts with α_{13}QL (Fig. 4E). Note that in one co-immunoprecipitation experiment (Fig. 4A), but not the reciprocal one (Fig. 4B), an apparent weak interaction between α_{13}QL and (32-912)p115 is observed, although it is not above the basal level of co-immunoprecipitation of wild type α_{13} and full-length p115-RhoGEF (Fig. 4A). A small degree of apparently activation-independent, or basal, interaction of wild type α_{13} and full-length p115-RhoGEF is typically observed in these co-immunoprecipitation assays (Fig. 4) [10]; this may represent wild type α_{13} binding to a non-RGS site on p115-RhoGEF or may reflect the presence of trace amounts of activating aluminum and fluoride in the samples. Importantly, the co-immunoprecipitation results using α_{13}QL further confirm that the N-terminal residues of p115-RhoGEF are critical for its binding to active α_{13}.

4. Discussion

In this report, we demonstrate that an acidic-rich region contained within the ~40 amino acid N-terminal extension of p115-RhoGEF is essential for binding to activated α_{13}. Deletion analysis determined that amino acids 22–31 encompassed a critical region for allowing p115-RhoGEF to interact with α_{13}, and, moreover, mutational analysis demonstrated that mutation of glutamic acids 27 and 29 together was sufficient to prevent p115-RhoGEF from binding to α_{13}, as measured in co-immunoprecipitation assays. In addition, we present the surprising result that α_{13} binding deficient N-terminal mutants of p115-RhoGEF retain α_{13}-mediated recruitment from the cytoplasm to PM, demonstrating a clear separation of these two activities of p115-RhoGEF: binding to activated α_{13} and translocation to the PM in response to activated α_{13}.

The studies described here, taken together with the recent in vitro studies using the isolated rgRGS domain reported by Wells et al. [10], define a novel aspect of the RGS domain of p115-RhoGEF compared to other RGS proteins. Although all RGS proteins share a conserved RGS core domain, they contain variable sequences and domains outside the RGS box [21] that influence subcellular localization and signaling specificity. However, for p115-RhoGEF, the N-terminal extension outside of the structurally defined rgRGS domain [17,18] appears to be required for interaction with its G protein binding partner, α_{13}. The exact mechanism of how the N-terminal acidic-rich region contributes to α_{13} binding is presently unclear. The acidic side chains may define a novel contact site for α_{13} or they may be necessary to maintain a conformation of the rgRGS domain that is competent to bind α_{13}. The

recently solved crystal structure of the rgRGS domain of p115-RhoGEF [17] utilized residues 42–252, and it was reported that the rgRGS domain failed to crystallize when it contained the N-terminal 41 amino acids. Thus, the N-terminal extension is likely flexible, at least in the absence of α_{13}, and is not intrinsically required for the stability of the rgRGS core domain. Interestingly, we note that a number of acidic residues are present immediately N-terminal to the rgRGS core domains of PDZ-RhoGEF and LARG, two other RhoGEFs that interact with α_{13}.

Although the mutants (42-912)p115, (32-912)p115 and p115(E27,29A) displayed very little or no interaction with either AlF_4^--activated α_{13} or the constitutively activated α_{13}QL, these mutants completely retained α_{13}-promoted membrane recruitment, as measured by immunofluorescence microscopy and subcellular fractionation. At least two alternative mechanisms, not necessarily mutually exclusive, may explain this apparent paradox. First, weak interactions between the rgRGS domain of p115-RhoGEF and α_{13}QL, not evident in co-immunoprecipitation experiments, may suffice to bring the N-terminal mutants to the PM where additional membrane localization mechanisms can compensate for decreased binding to α_{13}. For example, we recently demonstrated, by deletion analysis, that p115-RhoGEF's PH domain is crucial for strong PM targeting [7]; binding of the PH domain to membrane lipids or proteins may allow tight PM binding, even when interaction with α_{13} is disrupted as seen with the N-terminal mutants of p115-RhoGEF. Consistent with the possibility that p115-RhoGEF mutants retain a weak interaction with α_{13}, a domain of p115-RhoGEF consisting of amino acids 42–252 retains 10–20% of the α_{13} binding ability of a 1–252 rgRGS fragment [10], and, in some cases, we could detect a small level of co-immunoprecipitation of α_{13}QL and mutants such as (32-912)p115 (Fig. 4A). Alternatively, our results are consistent with the presence of an additional binding site for α_{13} on p115-RhoGEF. Recent reports have suggested the presence of such an additional interaction site [7,10] that is revealed only upon combined N- and C-terminal truncation of p115-RhoGEF. In this model, the secondary interaction site for α_{13} must function in concert with the rgRGS domain, since deletion of the complete N-terminus, consisting of the N-terminal extension and rgRGS domain, results in a p115-RhoGEF deletion mutant that is refractory to α_{13}-dependent PM recruitment (Fig. 3A, c and d) [7] and to α_{13} stimulation of Rho exchange activity in vitro [10]. Although the mechanistic basis remains unclear for α_{13} activation of the Rho exchange activity of p115-RhoGEF, the work presented in this report provides new insight into the determinants responsible for the interaction of α_{13} and p115-RhoGEF.

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