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Identification of Structural Determinants on Concertina Governing Binding to the Cytosolic Non-receptor Activator Ric-8

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Abstract

Heterotrimeric G proteins relay a variety of extracellular signals to downstream intracellular effector proteins. Activation of the G protein α subunit (Gα), a requisite for signal propagation, requires the external action of a guanine nucleotide exchange factor (GEF), typically a G-protein-coupled receptor, which stimulates the Gα subunit to exchange GDP for GTP by providing a stable nucleotide-free transition state. Recently, cytosolic Ric-8 proteins have been demonstrated to possess GEF activity and interact *in vitro* with different subsets of monomeric Gα subunits. Since a crystal structure of Ric-8 in complex with a GDP-bound or nucleotide-free Gα subunit is not available, our goal has been to characterize this structural interface in the *Drosophila* model system using a combination of scanning mutagenesis and co-precipitation assays. A comprehensive Gα12 NAAIRS library was used as a proxy to uncover structural determinates on Concertina (Cta), the *Drosophila* Gα12 homolog, governing binding to the Ric-8 N-terminus. Initial screening of this library has revealed two distinct regions of Gα12 critical for interaction with the N-terminus of Ric-8. Unfortunately, corresponding Cta mutants, assayed by our collaborators, failed to show any relative binding impairment to Ric-8, suggesting that perhaps the residues mutated are not functionally conserved.

1. Introduction

The protein Ric-8 was recently identified by Miller *et al.* through a functional genomic screen in *Caenorhabditis elegans* designed to reveal novel proteins involved in the process of synaptic transmission[1]. Ric-8 (resistant to inhibitors of cholinesterase 8) was found to be a conserved cytoplasmic protein involved in heterotrimeric G protein α-subunit (Gα) mediated neurotransmitter secretion and early *C. elegans* embryogenesis. To highlight this dual functionality, Ric-8 was coined "synembryn" [2]. Two mammalian homologs of *C. elegans* Ric-8/synembryn were subsequently discovered by Tall *et al.* during yeast two-hybrid screens to identify novel downstream Gα signaling factors. These homologs, termed Ric-8A and Ric-8B, interact *in vitro* with different subsets of Gα proteins. Biochemical characterization revealed Ric-8A to function as a potent, receptor-independent, guanine nucleotide exchange factor (GEF) for select monomeric Gα subunits. Ric-8A binds to GDP-bound Gα subunits in the absence of the G-protein βγ dimer, stimulating the release of GDP, and forming a stable nucleotide-free Ric-8A:Gα transition state complex. Subsequent binding by GTP to Gα dissociates this complex, releasing Ric-8A and the activated, GTP-bound, Gα subunit[3]. These conformationally activated Gα subunits, in the canonical model of G protein signaling, are capable of eliciting a variety of intracellular responses[4]. Most recently, Ric-8 proteins have been shown to act as molecular chaperones for nascent Gα subunits, mediating their initial translocation and association with the endoplasmic reticulum before their ultimate trafficking to the inner-leaflet of the plasma membrane. Consequently, it has been speculated that Ric-8 GEF activity may not be intended, or solely intended, for the purpose of eliciting signaling responses through activated Gα proteins, but rather, as a device to uncouple Ric-8

from Gα subunits following completion of Ric-8-assisted folding[5]. To date, the crystallographic structure of Ric-8 or Ric-8 in complex with a GDP –bound or a nucleotide-free Gα subunit has remained elusive. Bioinformatics approaches, though, have provided some structural insight offering that Ric-8 is an armadillo type protein composed of ten armadillo domains repeated in tandem from the N- to C-terminus[6]. Elucidation of the unknown structural interface governing the Ric-8:Gα interaction will permit future studies where selective uncoupling of this interaction could be exploited as a method to further understand the biological implications of Ric-8.

The impetus for our investigation stems from correspondence with Dr. Steve Rogers and his graduate student, Dr. Kim Peters, at the Univ. of North Carolina Chapel Hill, who found from a functional genomic screen in fruit flies (unpublished data), that Ric-8 is necessary for stimulation of contractility mediated by the *Drosophila* homolog of Gα12, Concertina (Cta). To further investigate the importance of Ric-8:Cta interaction in *Drosophila*, we exploited the homology in amino sequence between Gα12 and Cta, and used a comprehensive library of Gα12 NAAIRS cassette mutants to indirectly identify regions on Cta presumed as determinants of Ric-8 binding. Since we were unable to express full-length *Drosophila* Ric-8 in *E.coli*, this screening was performed with a C-terminally truncated Ric-8 construct, termed Ric8NT, encompassing only the first five N-terminal armadillo repeats. Thus far in our examination, we report the discovery of two distinct regions of Gα12 that are critical for Ric8NT interaction, and discuss communicated experimental results involving Cta mutants deficient in the corresponding residues.

2. Experimental Procedures

2.1. Creation of GST-Ric8NT

Drosophila Ric-8 cDNA in pET was kindly provided by Steve Rogers (Univ. of North Carolina Chapel Hill). Forward (fwd) and reverse (rv) oligonucleotides were engineered to amplify the nucleotide sequence encoding amino acids 1-236 of Ric-8 and to flank this amplicon with the restriction sites BamHI (upstream) and XhoI (downstream) to allow ligation into the GST-fusion vector pGEX-5x-1 (GE healthcare). To halt protein expression after M^{236}, which trails the sequence "NIICELLKV," a stop codon (TGA) was introduced using the rv primer. Further, the addition of two arbitrary nucleotides between the BamHI cut site (GGATCC) and the Ric-8 start codon (ATG) was necessary in the fwd oligonucleotide to maintain the correct reading frame for the expression of GST-tagged C-terminally truncated Ric-8. Below are the primers used in polymerase chain reaction (PCR):

5' CTC GGATCC TT ATG GAA ACG GAG CAC CTG AAA CG 3'

3' G ACG CTT GAC GAG TTC CAC TAC ACT GAGCTC TCC 5'

The following PCR cycle, repeated 27 times, was used for Ric8NT fragment generation and amplification: 95°C for 1 min, 94°C for 45 sec, 57°C for 1 min, and 72°C for 1 min 45 sec. A final elongation of 5 min culminated the final (28^{th}) cycle. Construct was verified by sequencing using vector specific 5' and 3' primers.

2.2. Gα12QL and Concertina mutagenesis

Wild type (Wt) N-terminally myc-tagged Concertina (Cta) and the constitutively active (Q303L) and inactive (G302A) variants were kindly provided by Kim Peters (Univ. of North Carolina Chapel Hill). All point mutations were introduced in existing cDNA constructs with oligonucleotides using a QuikChange II Site-Directed Mutagenesis Kit (Agilent), according to the manufacturer's specifications, with the following noteworthy modification: for each mutagenesis, fwd and rv primers were initially separated and allowed to proceed with the template for 2 cycles in independent half-reactions, before combination, and the execution of the remaining 15 cycles.

2.3. Expression and purification of GST-fusion proteins

Vectors harboring the DNA encoding GST-Ric8NT and GST were transformed into BL21-Gold(DE3) *E. coli* (Stratagene). Discrete colonies of each construct were used to inoculate 12 ml cultures of Luria-Bertani (LB) broth containing ampicillin (Amp) at a concentration of 75 μg/ml. Following incubation at 37°C for 12-16 h under 220 rpm of agitation, 6 ml of the turbid 12 ml LB-Amp cultures were used to inoculate respective 500 ml LB-Amp cultures which were then grown under identical temperature and agitation. After 90 min of incubation, and every 20 min thereafter if necessary, absorbance by the cultures at 600 nm was assessed spectrophotometrically. Once the A_{600} value read between 0.5 and 0.8, isopropyl-β-D-thiogalactopyranoside (Fisher Scientific) was added at a concentration of 0.5 mM to induce protein expression. Cultures were then afforded 3 h of additional growth. Bacteria were harvested by centrifugation at 6000 x *g*, 4°C. Cell pellets were homogenized in 2.5 ml of cold GST Buffer A (2.3 M sucrose, 50 mM Tris pH 7.7, 1 mM EDTA) supplemented with protease inhibitors. 10 ml of cold GST Buffer B (50 mM Tris pH 7.7, 10 mM KCl, 1 mM EDTA) supplemented with 1 mM dithiothreitol (DTT) and protease inhibitors was then added to the homogenized bacterial suspension and mixed. Following the addition of 4-5 mg of lysozyme (MP Biomedicals), bacterial samples were incubated on ice for approx. 1 h, being swirled briefly every 10 min. Following incubation, crude lysates received 175 μL of 10% sodium deoxycholate, 260 μL of 1 M $MgCl_2$, and 25 μL of 5 mg/ml DNAse I, were rocked briefly until a noticeable decrease in viscosity occurred, and then clarified by centrifugation for 40 min at 22,000 x g, 4°C. Resulting supernatant, as described[7], was added to 0.35 ml of glutathione-sepharose 4B (GE Healthcare), already washed 3-times in ice-cold T_{50}ED buffer (50 mM Tris pH 7.7, 1 mM EDTA, 1 mM DTT), and allowed 45 min for interaction at 4°C. The glutathione-sepharose immobilized proteins were then subjected to 4 wash cycles with T_{50}ED buffer supplemented with approx. 150 mM NaCl. Aliquots of affinity-purified, immobilized proteins were snap-frozen in liquid N_2 and stored at -80°C.

2.4. Preparation of Gα12QL mutants from membrane detergent extracts

Human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified Eagle medium (Mediatech) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics, and were maintained at 37°C under 5% ambient CO_2. For each point or NAAIRS mutant of GTPase-deficient myc-tagged Gα12, 10 μg of plasmid DNA was used to transfect a 10-cm dish of HEK293 cells, at approx. 90% confluence, using 15 uL of a sterile stock solution of 2 mg/mL polyethylenimine (PEI) in ddH_20 (gift from Steve Rogers). The DNA and PEI were briefly mixed with 0.5 mL of room-temp. DMEM, allowed to incubate for 20 min, and then fed drop-wise to cells. At 36-48 h post-transfection, as described[7], cells were rinsed with room-temp. phosphate-buffered saline (PBS), scraped off dish in 3 mL of fresh PBS, and pelleted at 800 x g. To each cell pellet, 0.5 ml of ice-cold Lysis Buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM DTT, 10 mM $MgSO_4$) supplemented with 1% (w/v) polyoxyethylene-10-lauryl ether (LPX) and protease inhibitors was added while simultaneously plunging the vessel housing the pellet into icy-water. Samples were mixed by inversion for approx. 30 min at 4°C and then centrifuged at 100,000 x g, 4°C, for 1 h. Clarified lysates were aliquoted, snap-frozen in liquid N_2, and stored at -80°C.

2.5. GST pulldown of Gα12QL mutants

For each mutant of Gα12QL assayed, 60 μL of clarified HEK293 cell lysate (prepared as previously described) was diluted to 800 μL using Lysis Buffer, lowering the LPX concentration to 0.075%. From this diluted lysate, 30 μL was set aside as "load" and the remainder was equally divided and incubated with glutathione-sepharose immobilized GST and GST-Ric8NT. After 150 min of incubation at 4°C under constant inversion, samples were pelleted at 1300 x *g*, and the supernatants were discarded. Sepharose beads and accompanying "pulldowns" were washed three times with 1 mL volumes of ice-cold modified Lysis Buffer containing 0.05% LPX and then resuspended and denatured in SDS sample buffer. To verify equal amounts of GST and GST-Ric8NT in all samples assayed for interaction, 5 uL of each denatured sample was resolved by SDS-PAGE, stained with Coomassie blue, destained, and photographed. Remainders of each sample, and the "load," were subjected to SDS-PAGE and transferred onto nitrocellulose filters. Immunoblots were blocked for a minimum of 8 h at 4°C in TBST (50 mM Tris pH 7.7, 150 mM NaCl, 0.05% Tween-20) supplemented with 5% (w/v) powdered milk (Laura Lynn), and then were incubated for 3-4 h at room-temp. with rabbit-derived anti-Gα12 antibody (Santa Cruz Biotechnology) at a 1:500 dilution in TBST+5% milk. Following three 10-min washes with TBST, alkaline phosphatase-conjugated anti-rabbit antibody (Promega) was applied at a 1:7500 dilution in TBST+5% milk, and allowed to incubate for 1 h. Three

additional 10-min washes with TBST were performed, and blots were developed colorimetrically using the AP substrates NBT/BCIP (Promega) according to the manufacturer's instructions. Quenched blots were documented using a Kodak Gel Logic 100 scanner and the net Gaussian intensities of bands were computed using Carestream Molecular Imaging software (New Haven, CT).

3. Results

A comprehensive panel of 62 NAAIRS cassette mutants[8] stretching the entire amino acid sequence of constitutively active Gα12 were screened to efficiently identify regions of Gα12 involved in interaction with the N-terminus of Ric-8. Consecutive sextuplets of amino acid sequence within Gα12 starting from the N-terminal methionine were substituted with the well-tolerated hexameric sequence, asparagine-alanine-alanine-isoleucine-arginine-serine (NAAIRS) to yield a series of mutants each deficient in the structural properties of the amino acids displaced[9]. This approach has been previous implemented successfully in projects to map the binding domains of Gα12 and the scaffolding Aα subunit of PP2A[10], and further, in recent work to identify regions of Gα12 critical for interaction with the cytoplasmic C-terminal tail of polycystin-1[11]. Activation of Gα12 was achieved mutationally via the substitution of glutamine at position 229 (dashed box) for leucine[12]. All mutants were engineered in myc-tagged Gα12QL to permit the distinction in GST pulldowns between pelleted endogenous Gα12 and transiently expressed recombinant Gα12. The myc-epitope, flanked by the linker sequence serine-glycine-glycine-glycine-glycine-serine (SGGGGS), was inserted in the α-helical domain of Gα12QL between proline 139 and valine 140 [8]. The alphabetical designations of the NAAIRS mutants are shown in Fig.1., along with the location of myc-tag insertion (red arrow). Due to the presence of the myc-tag within the hexameric amino acid stretch denoted W, a NAAIRS substitution was not made, and the native sequence persists.

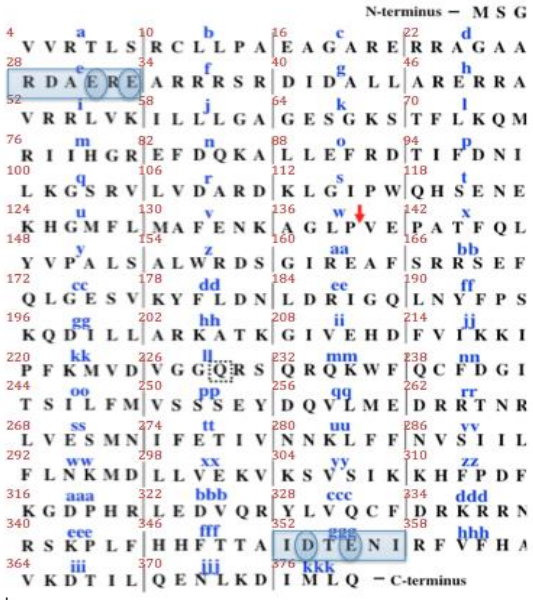

Figure 1. Schematic illustrating the location of NAAIRS and point mutants found to disrupt binding to the N-terminus of Ric-8. The alphabetical designations and residue locations of NAAIRS cassette mutants spanning the primary sequence of GTPase deficient Gα12 are shown[11]. Mutational activation of Gα12 was achieved by substitution of glutamine 229 (dashed box), for leucine. The red arrow denotes the point of insertion of a myc epitope tag, flanked by the linker sequence ser-gly-gly-gly-gly-ser, into Gα12.

Brief scanning of the NAAIRS library immediately revealed mutants E and GGG to be severely impaired in their ability to bind to the N-terminus of Ric-8. Lysates expressing these mutants, when incubated with GST-Ric8NT, yielded minimal pulldown while displaying, via the "loads," strong protein abundance (Fig. 2A). Calculation and subsequent comparison of the pulldown-to-load ratios of these mutants to the same measurement of the positive control, Gα12QL, reveals the relative numerical magnitude of their binding impairment, and is illustrated in Fig. 2B. Discovery of these critical hexameric binding locations in both the N- and C-termini of Gα12, prompted the creation

of specific point-mutants within the original displaced sequence to identify residues believed to be acting as binding determinants. Two negatively-charged, surface amino-acids, Asp 353 and Glu 355, within region GGG, were substituted simultaneously with positively-charged residues at the same locations, to generate a D353K/E355R point mutant. In addition, the identical charge substitutions were made individually in separate Gα12QL templates. Fortuitously, our lab already had built from other projects, a mutant within region E, where glutamic acid residues at positions 31 and 33 were replaced with arginine. Testing of these point mutants (DK/ER, D353K, and E31/33R) by previously employed co-precipitation assays (Fig. 2A), revealed the criticality of the displaced amino acids, in region E and GGG, in Ric8NT binding. Both double charge substitution mutants, DK/ER and E31/33R, drastically attenuated Gα12QL binding to Ric8NT, resulting in the loss of > 75% of interaction strength. In addition, the DK/ER sub-mutant, D353K, exhibited an approximate 50% loss of relative Ric8NT binding strength (Fig. 2B).

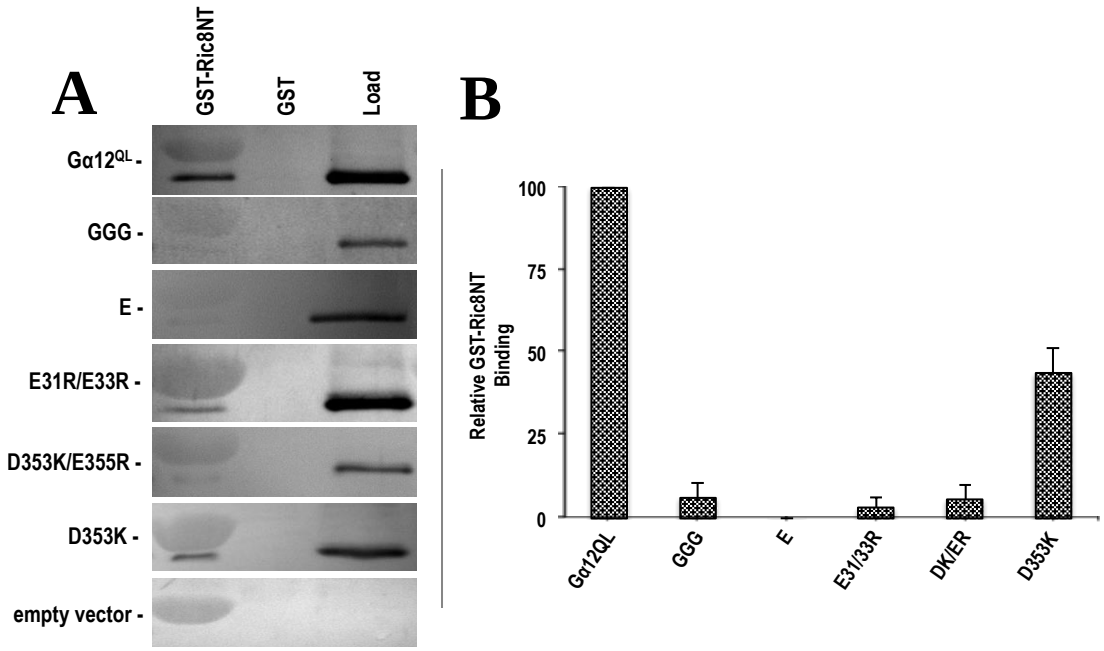

Figure 2. Specific regions and residues of Gα12 are critical for interaction with the Ric-8 N-terminus. (A) Representative Western blots of GST-Ric8NT pulldown experiments with myc-Gα12QL and myc-Gα12QL NAAIRS and point mutants. Lysates expressing either myc-tagged Gα12QL or each indicated mutant variant of the positive control (e.g. GGG or D353K) were prepared from HEK293 cells, and precipitated in separate reactions with GST-Ric8NT and GST alone as described in Section 2.5. The left and middle lanes of each panel display the pulldown of the indicated lysate by GST-Ric8NT and GST, respectively. The "load" in each panel (right lane) provides a measure of protein abundance within the tested lysate. Not shown, but available by request, are images of SDS polyacrylamide gels, stained with Coomassie Blue, which verify the equal distribution of GST and GST-Ric8NT beads among the lysates. (B) A bar graph illustrating the mean pulldown-to-load ratios of individual mutants as a percentage of the positive control (Gα12QL). Data shown represents a minimum of two independent experiments per lysate, and the standard error of each relative mean is reported.

4. Discussion

Residues corresponding to those in Gα12 which potently impaired Ga12QL binding to the Ric-8 N-terminus when displaced were identified in Concertina via a primary sequence alignment and subsequently substituted using the same charge mutations employed in Gα12. The mutations of E108/110R and D431K were each successfully engineered in wild-type and constitutively inactivated (G302A) N-terminally myc-tagged Concertina and sent to our collaborators, Dr. Steve Rogers and Dr. Kim Peters, at the Univ. of North Carolina Chapel Hill for evaluation in immuno-precipitation assays with full-length Ric-8. Results communicated by Dr. Peters (not included), show no

loss of relative Ric-8 binding by these mutants, and therefore suggest that the Gα12 residues found to be critical for Ric8NT binding are not functionality conserved from mammalian Gα12 to *Drosophila* Cta. This result, however, could be explained alternatively and may stem instead from additional Cta attachment points on full length Ric-8 that are not present in our engineered Ric8 truncation. Additionally, global misfolding of the activated Gα12 mutants, rather than the deficiency in the structural properties of amino acids displaced, could be responsible for the uncoupling of binding to Ric8NT. Currently, trypsin protection experiments involving these Gα12QL mutants are being undertaken as initially described by Kozasa and Gilman[13] to detect aberrant protein folding.

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6. References

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