Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Structural and functional analysis of FIP2 binding to the endosome-localised Rab25 GTPase

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ARTICLE INFO

Article history:
Received 27 May 2013
Received in revised form 8 September 2013
Accepted 12 September 2013
Available online xxxx

Keywords:
Rab25
FIP2
Rab11
Crystal structure
Effector
Endosome
Rab GTPase
Vesicle trafficking
Cancer

ABSTRACT

Rab small GTPases are the master regulators of intracellular trafficking in eukaryotes. They mediate spatial and temporal recruitment of effector proteins to distinct cellular compartments through GTP-induced changes in their conformation. Despite numerous structural studies, the molecular basis for Rab/effector specificity and subsequent biological activity remains poorly understood. Rab25, also known as Rab11c, which is epithelial-specific, has been heavily implicated in ovarian cancer development and independently appears to act as a tumour suppressor in the context of a distinct subset of carcinomas. Here, we show that Rab25 associates with FIP2 and can recruit this effector protein to endosomal membranes. We report the crystal structure of Rab25 in complex with the C-terminal region of FIP2, which consists of a central dimeric FIP2 coiled-coil that mediates a heterotetrameric Rab25-$(FIP2)_2$-Rab25 complex. Thermodynamic analyses show that, despite a relatively conserved interface, FIP2 binds to Rab25 with an approximate 3-fold weaker affinity than to Rab11a. Reduced affinity is mainly associated with lower enthalpic gains for Rab25:FIP2 complex formation, and can be attributed to subtle differences in the conformations of switch 1 and switch 2. These cellular, structural and thermodynamic studies provide insight into the Rab11/Rab25 subfamily of small GTPases that regulate endosomal trafficking pathways in eukaryotes.

1. Introduction

Rab GTPases are molecular switches that belong to the Ras protein superfamily and regulate vesicle trafficking in eukaryotic cells [1]. In their active GTP-bound state, membrane-localised Rabs recruit effector proteins to distinct sub-cellular compartments to exert their biological effects. Membrane attachment is facilitated by prenylation of one or two C-terminally situated cysteine residues via the enzyme geranylgeranyltransferase II (GGTase II; [2]). Upon hydrolysis of GTP, which is stimulated by GAPs (GTPase-activating proteins) [3], prenylated Rabs are extracted by GDI (GDP dissociation inhibitor) into the cytosol [4]. GDI can then deliver the GDP-bound Rab to an appropriate membrane. The precise details of how Rab proteins achieve association with the appropriate membranes are currently an issue of debate in the field (for recent reviews see [5–8]). Upon membrane delivery by GDI, the 'G-protein switch' is turned 'on' by GEFs (guanine nucleotide exchange factors) which promote the exchange of GDP for GTP, and the re-inserted Rab is primed for a new cycle of activity.

Rab structures comprise a 6-stranded mixed β-sheet flanked by five α-helices [9]. The 5′ phosphate arm of the nucleotide is bordered on one side by the P-loop (Walker A motif), which is conserved in the Ras superfamily, as well as in many ATPases [9,10]. The conformations of a pair of adjacent regions termed switch 1 and switch 2 are sensitive to the presence of GTP vs. GDP [11], and thus encode the nucleotide specificity for effector recruitment [12]. Additionally, a loop and strand situated between the switch motifs – termed the interswitch – also plays a role in effector recruitment. In particular, an invariant tryptophan residue that is located within an otherwise variable interswitch sequence provides a hydrophobic surface for effector binding [13]. The overall sequence identities between mammalian Rabs range from 33% to 78%, but the nucleotide-proximal motifs are the most highly conserved segments [14].

The complexity of Rab regulation of cellular trafficking originates from the extent of Rab/effector interactions in eukaryotic cells. Typically, a single Rab can bind to multiple effectors, often unrelated, while a single effector protein can sometimes be recognised by multiple Rab proteins. For example, Rab6 regulates Golgi traffic and interacts with unrelated effector proteins such as the Golgin GCC185 and DENND5

Abbreviations: ERC, endosomal-recycling compartment; FIP2, Rab11 family interacting protein 2; GAP, GTPase-activating protein; GDI, GTP/GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; GTP, guanosine 5′ triphosphate; ITC, isothermal titration calorimetry; MBP, maltose-binding protein; RBD, Rab11-binding domain

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http://dx.doi.org/10.1016/j.bbapap.2013.09.005

(alternatively named Rab6-interacting protein 1) [15,16]. The structures of binary complexes of Rab6/GCC185 and Rab6/R6IP1 have revealed that the former is a dimeric coiled-coil that recruits two Rabs, while the latter is a monomeric bundle of seven α-helices. However, the Rab6 interface is found along two parallel α-helices in both complexes, which have similar hydrophobic and hydrophilic features at their respective interfaces. Another example is Rab4 and Rab22, which bind to distinct segments of Rabenosyn-5 that, nevertheless, have a conserved 3-dimensional fold consisting of a coiled-coil of two anti-parallel α-helices [17]. In the majority of known complexes, binding is largely dependent on switch 1, switch 2 and the interswitch regions (reviewed in [18,19]), although additional nucleotide-insensitive parts of Rabs can also contribute to effector recruitment [20–23]. Structural studies of Rab-effector complexes have revealed that specificity is influenced by subtle differences in sequence and conformational diversity in switch 1, switch 2, the interswitch region, and residues bordering the switch/interswitch [13,17,24]. Even non-interacting residues in the hydrophobic core apparently influence the conformation of directly-interacting motifs [25].

Thermodynamic and kinetic studies of Rab/effector complexes reveal a variety of affinities in vitro ranging from relatively tight (Rab27/Slp2a, dissociation constant $K_d = 13$ nM) to weak (Rab1, Rab4, Rab5 and Rab8 complexes with OCRL, $K_d = 1–5$ μM) [20,26]. Despite numerous structural and thermodynamic studies of Rab/effector complexes, it is generally difficult to discern why some complexes have higher affinity than others, or whether the differences in affinity are meaningful (for a recent review see [19]). Closely associated with this issue is the question of the molecular determinants of Rab/effector specificity. Relating the structural data to the thermodynamic properties of complexes is essential for a thorough understanding of Rab/effector specificity, as well as the biological effects elicited by the complexes.

Rab25 (also known as Rab11c) was initially isolated from parietal cells and, unlike the closely related Rab11a and Rab11b proteins, its expression was found to be restricted to epithelial tissue [27,28]. Like all Rabs, Rab25 functions through the recruitment of downstream effector proteins, at least some of which it shares with Rab11a/Rab11b, which include a conserved protein family known as the FIPs (Rab11-family interacting proteins) [28,29]. Unique among the Rab family, wild-type Rab25 possesses a WDTAGLE sequence in the GTP-binding region of the protein (P3 phosphate-binding motif) while all other wild-type Rabs contain a WDTAGQE sequence [27]. In functional studies, the typical dominant-active Rab mutants employed have a Gln → Leu mutation at amino acid residue ~65–75. At this point, it is unclear what the biological significance, if any, of the naturally occurring WDTAGLE sequence in Rab25 is. In recent years, considerable evidence has emerged implicating Rab25 in a variety of cancers (for a recent review see [30]). Rab25, which is amplified in greater than 66% of breast and 80% of ovarian carcinomas, can determine the aggressiveness of some breast and ovarian cancers, and ovarian cancer patients with elevated Rab25 expression have markedly poorer outcomes [31]. At the cellular level, Rab25 drives invasive migration of ovarian cancer cells by regulating the trafficking of the adhesion molecule α5β1 integrin [32]. Rab25 also drives invasive migration of ovarian cancer cells by rescuing lysosomally-targeted integrins from degradation and transporting them back to the cell surface in conjunction with a protein known as CLIC3 (chloride intracellular channel protein 3) [33]. Intriguingly, in other cellular contexts including colon, oesophageal, head and neck and some breast carcinomas, Rab25 appears to function as a tumour suppressor [30,34–39]. Together these reports indicate that Rab25's involvement in cancer development is likely influenced by tissue/cell-specific regulators of cell polarity, adhesion and signalling.

Despite numerous studies linking Rab25 to cancer development and progression, there currently exists no cellular or structural data for Rab25-mediated recruitment of downstream effector proteins. Here, we provide evidence that Rab25 associates with FIP2 and can recruit this effector protein to endosomal membranes. We have also determined the crystal structure of Rab25, which contains a naturally occurring leucine residue in place of the typical catalytic glutamine, in complex with FIP2 at 1.8 Å resolution. Given that previously structures of Rab11:FIP2 and Rab11:FIP3 complexes contained a Rab11(Q70 → L) mutation to trap the GTP state [24,40,41], we also undertook the crystal structure determination of wild-type Rab11 in complex with FIP2 at 2.0 Å resolution. Comparisons of wtRab25:FIP2, wtRab11:FIP2 and the previously determined Rab11(Q → L):FIP3 complex provide an opportunity to understand the thermodynamic and molecular determinants of Rab-mediated effector recruitment.

2. Materials and methods

2.1. Plasmid construction

pEGFP-C1/Rab25 (full-length) was generated by PCR using the following primers (*Fwd* gcgaagcttcggggaatggaactgaggaagattat, *Rev* gcgg aattccgtcagaggctgatgcaacaggccct) with pcDNA3-HA/Rab25 (kind gift from Jim C. Norman) as template and cloned into the pEGFP-C1 vector (Clontech Laboratories, Inc.). pEGFP-C1/Rab25 I45E was generated by SDM (site-directed mutagenesis) using the following primers (*Fwd* gacagccgcaccaccgaggggttgagttctcc, *Rev* ggagaactcaacccctcggtggtgcgg ctgtc) with pEGFP-C1/Rab25 as template. pEGFP-C3/Rab11a was described previously [42]. pEGFP-C2/Rab11b was generated by subcloning the *BglII-XhoI* fragment from pACT2/Rab11b [plasmid isolated from a yeast two-hybrid screen (unpublished data)], into the pEGFP-C3 vector (Clontech Laboratories, Inc.). pEGFP-C1/FIP2 and pEGFP-C1/FIP2 I481E have been previously described [24,43]. pGBKT7/Rab11a was generated by sub-cloning the Rab11a fragment from pLexA/Rab11a (described in [44]) into pGBKT7 (Clontech Laboratories, Inc.). pGBKT7/Rab11b was generated by PCR using the following primers (*Fwd* gccgaattcatggggac ccgggac, *Rev* gcgggatcctcacaggttctgcag) with pEGFP-C2/Rab11b as template and cloned into pGBKT7. pGBKT7/Rab25 was generated by sub-cloning the Rab25 fragment from pEGFP-C1/Rab25 into pGBKT7. pGBKT7/Rab25 CC209-210SS was generated by SDM using the following primers (*Fwd* gggagaagagggccagtagcatcagcctctga, *Rev* tcagaggctgatgc tactggccctcttctccc) with pGBKT7/Rab25 as template. Cloning of pMAL/$FIP2_{410–512}$ has been described previously [24,45]. To generate pET28/$Rab25_{7–180}$, $Rab25_{7–180}$ was synthesised and codon-optimised for *Escherichia coli* expression (Geneart AG) and inserted into the NdeI/EcoRI site of a pET28 vector. pGADT7/RCP(FIP1C) was generated by PCR using the following primers (Fwd cccgaattcatgtccctaatggtctcggctggc, Rev cccgaattcttacatctttcctgcttttttgcc) with pEGFP-C3/RCP [46] as template and cloned into the pGADT7-AD vector (Clontech Laboratories, Inc.). pGADT7-AD/Rip11 was generated by PCR using the following primers (Fwd cccgaattcgccctggtgcggggcgcggag, Rev cccgaattcctatttg gggggcccgggg) with pBluescript/KIAA 0857 (Kazusa) as template and cloned into the pGADT7-AD vector. pGADT7-AD/FIP2 was generated by PCR using the following primers (Fwd cccgaattcatgatgctgtccgag caagcccaaaagtg, Rev cccgaattcttaactgttagagaatttgccagctttc) with pEGFP-C1/FIP2 as template and cloned into the pGADT7-AD vector. pGADT7-AD/FIP2 I481E was generated by PCR using the following primers (Fwd cccgaattcatgatgctgtccgagcaagcccaaaagtg, Rev cccgaattcttaactgttagagaa tttgccagctttc) with pEGFP-C1/FIP2 I481E as template and cloned into the pGADT7-AD vector. pGADT7-AD/FIP3 was generated by sub-cloning FIP3 from pGBKT7/FIP3 into pGADT7-AD. pGADT-AD/FIP4 was generated by PCR using the following primers (Fwd cccgaattcgcg ggcggcgcgggctggcg, Rev cccgaattcttagtgtttgatctcgaggatg) with pEGFP-C1/FIP4 [47] as template and cloned into pGADT7-AD. pTrcHisC/$FIP2_{302–512}$ was generated by PCR using the following primers (Fwd cccggatccaagacccatttacaaatgtgactg, Rev cccgaattcttaactgttagagaatttgccag) with pEGFP-C1/FIP2 as template and cloned into the pTrcHisC vector (Invitrogen Corporation). All constructs generated by PCR were verified to be correct by double strand DNA sequencing (Macrogen).

Please cite this article as: P. Lall, et al., Structural and functional analysis of FIP2 binding to the endosome-localised Rab25 GTPase, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbapap.2013.09.005