The Identification and Characterisation of Rab4 Interacting Proteins

Thesis submitted for the degree of

Doctor of Philosophy

By

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Submitted: December 2001

To my Family

In theory, there is no difference between theory and practice.
But in practice, there is.

Jan L.A. van de Snepschuet

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Declaration

I hereby declare that this thesis presented for the degree of Doctor of Philosophy to the National University of Ireland has not previously been presented for a higher degree to this, or any other University. Any assistance provided is acknowledged in the text by reference to the researchers or their publications. This thesis is of my own compostion and may be photocopied or lent to other libraries for the purpose of consultation.

Andrew J. Lindsay, BA Mod (Genetics).

Abbreviations

ADP	Adenosine 5’ diphosphate
AP	Adaptor protein complex
ARF	Adenosine ribosylation factor
ATP	Adenosine 5’ triphosphate
bp	Base pair
BSA	Bovine serum albumin
CCV	Clathrin coated vesicle
CMV	Cytomegalovirus
Ct	Carboxy-terminus
DLIC	Dynein light intermediate chain
DMEM	Dulbecco’s Modified Eagles Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
EST	Expressed sequence tag
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FL	Full-length
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine 5’ diphosphate
GEF	Guanine nucleotide exchange factor
GLUT	Glucose transporter
GTP	Guanine 5’ triphosphate
HEPES	N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
IPTG	Isopropylthio-β-galactosidase

kb	Kilobase pairs
kDa	Kilodaltons
LBPA	Lysobisphosphatidic acid
LDL	Low density lipoprotein
MOI	Multiplicity of infection
$MgCl_2$	Magnesium chloride
MTOC	Microtubule organising centre
NaCl	Sodium chloride
NSF	N-ethylmaleimide sensitive factor
Nt	Amino-terminus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PtdIns3*P*	Phosphatidyl inositol-3-phosphate
PI(3) kinase	Phosphatidyl inositol-3-phosphate kinase
PNS	Postnuclear supernatant
RC	Recycling compartment
REP	Rab escort protein
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor protein
TGN	*Trans*-Golgi protein
Tfn	Transferrin
TfnR	Transferrin Receptor
TxR	Texas Red
WT	Wild-type
X-Gal	5-bromo, 3-Chloro, 2-Inodyl, β-D-thiogalactosidase

Abstract

Rab4 is a member of the Rab superfamily of small GTPases. It is localized to the early sorting endosome and plays a role in regulating the transport from this compartment to the recycling and degradative pathways. In order to further our understanding of the role Rab4 plays in endocytosis, a yeast two-hybrid screen was performed to identify putative Rab4 effectors. A constitutively active mutant of Rab4, Rab4Q67L, when used as bait to screen a HeLa cDNA library, identified a novel 80kDa protein that interacted with Rab4-GTP. This protein was called Rab Coupling Protein (RCP).

RCP interacts preferentially with the GTP-bound form of Rab4. Subsequent work demonstrated that RCP also interacts with Rab11, and that this interaction is not nucleotide-depenedent. RCP is predominantly membrane-bound and localised to the perinuclear recycling compartment. Expression of a truncation mutant of RCP, that contains the Rab binding domain, in HeLa cells, results in the formation of an extensive tubular network that can be labelled with transferrin. These tubules are derived from the recycling compartment since they are inaccessible to transferrin when the ligand is internalised at 18^{o}C. The truncation mutant-induced morphology can be rescued by overexpression of active Rab11, but not active Rab4. This suggests that RCP functions between Rab4 and Rab11 in the receptor recycling pathway, and may act as a 'molecular bridge' between these two sequentially acting small GTPases. Quantitative assays demonstrated that overexpression of the truncation mutant results in a dramatic inhibition in the rate of receptor recycling.

Database analysis revealed that RCP belongs to a family of Rab interacting proteins, each characterised by a carboxy-terminal coiled-coil domain and an amino-terminal phospholipid-binding domain. KIAA0941, an RCP homologue, interacts with Rab11, but not with Rab4. Overexpression of its Rab binding domain also results in a tubular network, however, this tubulation cannot be rescued by active Rab11.

1 Introduction

The eukaryotic cell has evolved an intricate machinery to transport cargo. There are numerous directions in which such cargo has to be transported. In broad terms these directions include into, out of, and within, the cell. However, each of these transport pathways can be subdivided into a series of highly regulated steps. The necessity to maintain the integrity and specificity of the multitude of transport steps requires a highly dynamic, and elaborate, machinery. Cargo is transported to and from the plasma membrane, and between organelles, in membrane-bound vesicles. The integrity of these transport processes is preserved by large protein complexes, unique to each step, that are present on donor and target vesicles. The precise mechanism of even one of these steps is still not fully understood. However, many of the important components involved have been identified. Key among these are the SNARE proteins and the small GTPases. The SNARE proteins mediate the fusion of transport vesicles with their target membranes. Several families of small GTPase, belonging to the Ras superfamily of small G-proteins, are involved in regulating the transport of cargo around the cell. ADP-ribosylation factor (ARF) GTPases are involved in maintaining the integrity of organelles and in the process of intracellular traffic. Rab GTPases are localised to the cytoplasmic face of all organelles involved in intracellular transport, where they play important regulatory roles in all aspects of each transport route. A family of larger GTPases, the dynamins, have been implicated in a process known as budding, during which pits containing cargo form in the plasma membrane and ultimately pinch off into the cytoplasm, forming vesicles.

Intracellular transport can be divided into two main pathways, the endocytic and secretory pathways. The secretory pathway comprises the endoplasmic reticulum (ER), the Golgi apparatus, the vacuole, and the vesicles that mediate transport between them. A protein that enters the secretory pathway is destined to undergo one of several possible fates, depending on the sorting signal it carries. A secretory protein, after passing through the ER and Golgi, is packaged into a secretory vesicle that is transported to, and fuses with, the plasma membrane resulting in the release of the protein into the extracellular space. Other proteins, instead of being secreted, may possess sorting information that specifies transport to a specific intracellular location, such as the vacuole. Finally some proteins contain retention signals that result in their permanent localisation in an organelle of the secretory pathway. Such proteins provide catalytic activities necessary for the function of the organelle e.g. the mannosyltransferases that reside in the Golgi apparatus.

The endocytic pathway can be subdivided into clathrin-dependent and clathrin-independent endocytosis. The clathrin-dependent pathway is the better understood of the two and is the focus of this thesis. The clathrin-independent mechanism of endocytosis was initially observed in a mammalian system where dynamin function was switched off. This resulted in the rapid reduction of both receptor-mediated and fluid-phase endocytosis. However, within an hour fluid uptake returned to normal while receptor uptake remained inhibited (Mellman, 1996). Since internalisation of fluid markers was restored, despite the downregulation of clathrin-dependent endocytosis, another pathway must exist that can partially compensate for the inhibition. Further work identified caveolae, plasma membrane pits lacking clathrin, as a possible means for fluid uptake. Caveolae are found at varying frequencies in different cell types. They are most prominent in adipocytes, fibroblasts, and muscle cells. They have a characteristic striated appearance due to the accumulation of the integral membrane protein, caveolin. Caveolae have been implicated in endocytosis, transcytosis, and molecular sorting. At the plasma membrane they also serve to compartmentalise and integrate a wide variety of signal transduction pathways.

1.1 Receptor-Mediated Endocytosis

Receptor-mediated endocytosis is a process by which molecules present in the extracellular medium bind to specific receptors, located on the cell surface, and are subsequently transported to predetermined intracellular compartments. Receptors on the plasma membrane when bound by their ligand cluster together in small indentations, or invaginations, on the plasma membrane. These invaginations become coated by clathrin, which forms a lattice around the pit that acts as a scaffold for the recruitment of adaptor proteins. The adaptor proteins recognise signal sequences present on the cytoplasmic domain of receptors destined for internalisation (Robinson and Bonifacino, 2001). Dynamin wraps around the neck of these pits, which grow deeper and deeper into the cytoplasm, and is involved in their fission, or “pinching off”, to form clathrin coated vesicles (CCVs) (Hinshaw, 2000). These newly formed CCVs are transported to the early/sorting endosomes (SE). The pH difference in the sorting endosome results in the dissociation of the ligand/receptor complex. The cargo is then transported along the late endocytic pathway, eventually ending up in the

lysosomes where it is broken down. Meanwhile, the receptor can be recycled directly back to the plasma membrane from the sorting endosome, along the fast recycling pathway, or it may enter the pericentriolar recycling compartment (RC), prior to transport back to the plasma membrane. This is termed the slow recycling pathway. In some cases, the receptor follows its cargo along the late endocytic pathway to be broken down in the lysosomes.

The transferrin receptor (TfnR) is a classic example of a receptor that follows the recycling pathway. It is a constitutively endocytosed receptor, i.e. it is endocytosed whether or not it is bound by its ligand (Willingham and Pastan, 1985). On the cell surface it can be bound by its ligand, iron-saturated transferrin (Tfn). The receptor is brought into the cell in CCVs, the iron dissociates in the SE and is transported to the lysosomes via the late endosome (LE). Meanwhile, the Tfn/TfnR complex is recycled back to the plasma membrane via the recycling compartment (Dautry-Varsat, 1986). The internalisation signal on the transferrin receptor is constitutively accessible to the adaptor complexes. In contrast, the epidermal growth factor (EGF) receptor carries a 'cryptic' internalisation signal that only becomes accessible to adaptors when it is bound by its ligand. Upon ligand binding, the EGF receptor undergoes tyrosine kinase activation, and autophosphorylation, that results in a structural change of the receptor's cytoplasmic domain, exposing the internalisation signals. When internalised the EGF receptor follows a different pathway to the transferrin receptor. At least 50% of the EGF receptor, when bound by EGF, is transported to the lysosomes for degradation, via the sorting and late endosomes (see Figure 2) (Mellman, 1996).

1.1.1 Clathrin-Coated Vesicles

The use of clathrin coats is one of the mechanisms by which the cell forms vesicles. Clathrin is a coat protein. Three coat complexes have been well characterised, clathrin, coat protein-I (COP-I), and coat protein-II (COP-II) (Glick and Malhotra, 1998). COP-I coats are found on vesicles that travel between the Golgi apparatus and the endoplasmic reticulum, and also between the Golgi and late endosomes. COP-II coats are also involved in transport from the ER to the Golgi.

Clathrin coats are involved in transport of vesicles from the plasma membrane to early endosomes, and from the *trans*-Golgi network (TGN) to endosomes.

One of the early steps of endocytosis is the assembly of clathrin coats on the cytoplasmic face of the plasma membrane resulting in the formation of invaginated pits that eventually "pinch off" (scission) to become independent CCVs. Clathrin coats are primarily made up of an approximately 190kDa clathrin heavy chain (CHC) and a 25kDa clathrin light chain (CLC). These associate into CHC-CLC complexes that form three-legged trimers, known as triskelions. The triskelions oligomerise to form polygonal arrays that can form into a clathrin cage around lipid-vesicles. Pearse and colleagues used electron microscopy (EM) to determine the first structural map of a clathrin cage, which contains eight hexagons and twelve pentagons (thirty-six triskelions) (Crowther and Pearse, 1981; Smith *et al.,* 1998).

Other components of the clathrin coat include assembly protein 180 (AP180) and the adaptor proteins (APs). AP180 has been shown *in vitro* to have assembly properties i.e. promotes the formation of clathrin coats. Adaptor proteins are the first to be recruited to membranes and provide the binding site for clathrin coats. Their other crucial function is to interact with cell surface receptors that carry specific target signals that program them for sorting into CCVs. As with the coat proteins, there are several adaptor protein complexes. Each complex contains four polypeptides (adaptins), two 100kDa heavy chains, a 50kDa μ (medium) chain, and a small 25kDa σ chain (Figure 1). The two heavy chains have appendages, known as ear domains, that protrude from the complex and to which several proteins involved in endocytosis have been shown to bind, these include amphiphysin, Eps15, and epsin. Genomic analysis of the adaptor complexes in a number of eukaryotic organisms has revealed that while the number of complexes remains relatively constant from yeast to human, the number of subunits has increased, from 31 in yeast to 53 in human (Bock *et al.,* 2001). Thus, instead of evolving new adaptor complexes to accommodate the increased complexity of the trafficking system, mammals have adapted by exchanging subunits. For example, the AP-1 complex can interchange between two medium subunits, but only μ1B can transport low-density lipoprotein and transferrin receptors. The AP-1 complexes are found associated with CCVs derived from the TGN, and their chains are designated γ, β1, μ1, and σ1. AP-2 complexes are associated with CCVs originating from the plasma membrane and have polypeptides designated

α, β2, μ2, and σ2 (Robinson, 1994). Other adaptor complexes include AP3 and AP4, however, these are not as well characterised (Hirst *et al.,* 1999; Simpson *et al.,* 1997);

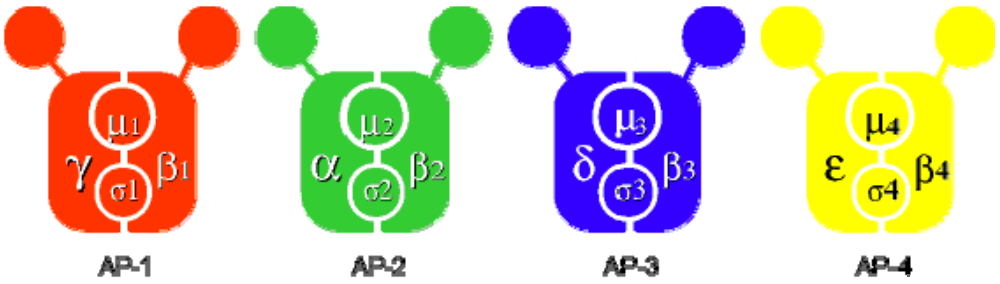

Figure 1. Schematic representation of the four known adaptor complexes (www.cimr.cam.ac.uk/cellbiol/robinson).

In addition to the core scaffold proteins of clathrin coats, there are many other proteins that can bind clathrin or adaptor complexes. These are thought to play roles in modulating cage assembly, its disassembly, and the eventual recycling of the coat components. One of the proteins involved in the scission event is the GTPase dynamin. In *Drosophila* that carry the *Shibire* mutation, a temperature-sensitive mutation in dynamin, and in cells that express a GTPase defective form of dynamin, clathrin coated pits appear to accumulate on the plasma membrane with very little CCV formation. Dynamin can also be seen on the necks of deeply invaginated pits by EM. It is likely that dynamin may constrict the neck of emerging vesicles, and in conjunction with the closing of the clathrin cage, result in the pinching off of the newly formed vesicle from the plasma membrane. However, dynamin is not exclusively involved in budding of CCVs from the plasma membrane, there are at least twenty isoforms of dynamin, some of which have been implicated in Golgi function, caveolae formation, and mitochondrial biogenesis (Schmid *et al.,* 1998).

1.1.2 The Early Sorting Endosome and Recycling Compartment

The early sorting endosome is the 'junction' between the recycling and the degradative pathways. The physical structure of the early endosome is complex, consisting of large vesicles (300-400nm in diameter) from which thin tubules (approx. 60nm in diameter) emanate. The vesicles contain invaginations and closely resemble

the multivesicular bodies (MVBs) of the degradative pathway, while the tubular elements resemble the tubules of the recycling compartment. While the boundary between the early sorting endosome and the late endosomes can be readily distinguished, in some cell lines it is difficult to determine a boundary between the recycling compartment and the early sorting endosome. It is in the early sorting endosome that proteins and lipids, internalised from the plasma membrane, are sorted into cargo that is to be degraded, or recycled. Cargo from the early sorting endosome is either transported directly to the plasma membrane, in the 'fast' recycling pathway, or sent to the recycling compartment for recycling along the 'slow' pathway. Sorting motifs have been found on the cytoplasmic domains of a number of proteins that are destined for the degradative pathway, including the EGFR, P-selectin, and the IL-2 receptor. However, no recycling motifs have been identified, leading to the suggestion that recycling to the cell surface occurs by default. The lipid composition of the sorting endosome is not uniform, which may provide a means by which proteins in the early endosome are segregated. Different lipid domains, with associated cargo, may accumulate at the regions of tubule or MVB formation, depending on their fate (Gruenberg, 2001). Lipid rafts, assemblies of cholesterol and sphingolipids, may have sorting roles in the early endosome.

The recycling compartment is composed of distinct tubules that are generally found close to the microtubule organising centre (MTOC). They are slightly less acidic than sorting endosomes and do not contain ligands or receptors destined for the degradative pathway. Traffic has been observed from the recycling compartment to the plasma membrane (the 'slow' recycling pathway), and also from the recycling compartment to the *trans*-Golgi network (Ghosh *et al.,* 1998). Transport from the recycling compartment is dependent on microtubules, actin, and unconventional myosins. There is considerable overlap in the distribution of the key components that regulate transport to and from the sorting endosomes and recycling compartment. For example, immunofluorescence, using fluorescent-tagged proteins, and biochemical techniques that use immunoadsorption and centrifugation to separate the two compartments, have clearly shown that there is significant overlap in the distribution of three Rab GTPases (Rabs 5, 4, and 11) involved in the receptor-recycling pathway (Sonnichsen *et al.,* 2000; Trischler *et al.,* 1999). Rab5, which regulates transport from the plasma membrane to the early endosome, shows some overlap with Rab11, which regulates transport through the recycling compartment. Even though there is overlap,

these Rab proteins interact with specific subsets of effectors that appear to be more precisely localised to the compartment upon which they function. Rab4 appears to function in between Rab5 and Rab11 in the recycling pathway. It regulates transport from the sorting endosome to the recycling compartment, or to the degradative pathway (McCaffrey *et al.,* 2001; van der Sluijs *et al.,* 1992), and shares an effector with Rab5, Rabaptin-5 (Vitale *et al.,* 1998). It has been speculated that there is a similar protein that acts as an intermediate between Rab4 and Rab11 (Sonnichsen *et al.,* 2000).

Note:-the nomenclature used to describe the compartments along the recycling pathway can be confusing. When referring to the early endosome, some authors describe it as being composed of both the sorting endosome and the recycling compartment, while others describe the early endosome as the equivalent to the sorting endosome, and distinct from the recycling compartment, or recycling endosomes.

1.1.3 The Degradative Pathway

Cargo that is destined to travel along the degradative pathway is transported to the late endosomes, from the early sorting endosome, in large multivesicular bodies. It is not clear if MVBs are transport intermediates between two stable compartments, or if they eventually mature into late endosomes. Mutivesicular bodies consist of two membranes, an outer limiting membrane, and an internal membrane with many invaginations. The outer membrane does not contain any early endosomal markers, and lacks LAMP1, which is abundant on late endosomal membranes. Similar to early endosomes, late endosomes are highly pleiomorphic, containing a cisternal region, tubules, and vesicular regions with invaginations. Their limiting membranes contain high quantities of the protein LAMP1, which is protected from degradation by its glycosylation state. The inner membrane is partially composed of the phospholipid, lysobisphosphatidic acid (LBPA). LBPA is resistant to degradation by phospholipases. It has been proposed that LBPA presents proteins and lipids to the degradation machinery. Mannose 6-phosphate receptors (M6PRs) travel from the TGN to late endosomes, where they deliver lysosomal enzymes. The distinction between late endosomes and lysosomes is even more blurred than that between early

sorting endosomes and late endosomes. They both have an approximate pH of 5.5, and their outer membranes are primarily composed of the same glycoproteins. They share the same docking/fusion machinery and can rapidly exchange phospholipids and membrane proteins. However, lysosomes are more electron-dense than late endosomes, and lack M6PR, Rab7, and Rab9, or phosphorylated hydrolase precursors (Gruenberg, 2001).

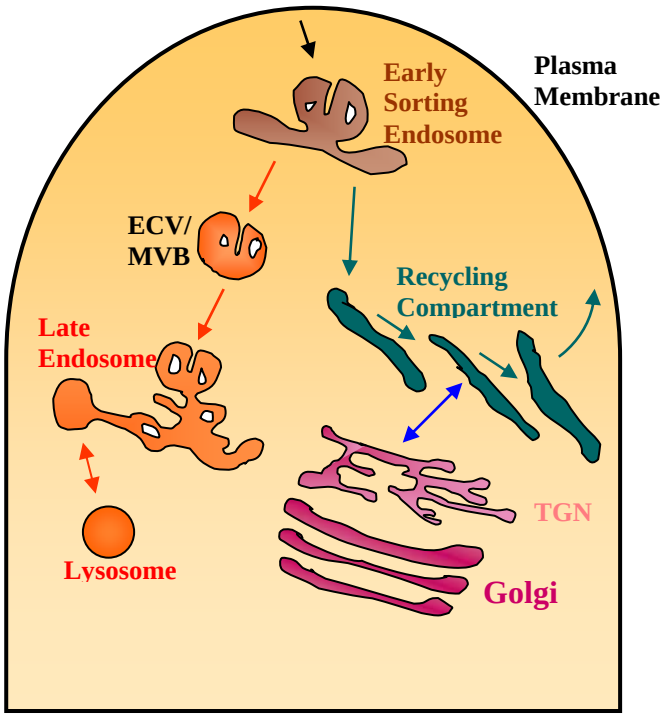

Figure 2. Outline of the endocytic pathway. The recycling pathway is indicated by green arrows, and the degradative pathway by red arrows. The blue arrow represents transport between the TGN and the recycling compartment.

1.2 The SNARE Hypothesis

SNAREs, or SNAP receptors, are a family of integral membrane proteins that are involved in mediating the fusion of vesicles. They are generally between 15 and 45kDa in size. They are anchored to their specific membrane via their carboxy-terminus, while their central region and amino-termini are cytoplasmic. Specific subsets of SNAREs found on the target membrane (t-SNAREs) interact with specific SNAREs on the vesicle membrane (v-SNAREs). Coiled-coil domains in their

cytoplasmic region mediate these protein-protein interactions. SNAREs are specific to particular transport pathways, and some are also cell-type specific. Syntaxins are a family of t-SNAREs, syntaxin 1 is a neuronal-specific t-SNARE, while syntaxin 2 is found at the apical membrane of epithelial cells. Synaptobrevin 3 (also known as cellubrevin) is an example of a ubiquitously expressed v-SNARE that localises to the perinuclear recycling compartment.

The involvement of SNAREs in membrane fusion can be described as a series of distinct steps. Taking the t-SNARE syntaxin 1 as an example, initially it is in a 'closed' conformation. This conformation is maintained by a specific alpha-helix, the H3 helix, which is bound by a three helix bundle (the inhibitory domain) located at the syntaxin amino-terminus. The resulting four-helix bundle cannot bind its cognate v-SNARE. Step 1 involves the conversion of syntaxin 1 from its 'closed' conformation to an 'open' conformation. In step 2, the v-SNARE binds to the H3 helix of syntaxin and also to a second t-SNARE. This results in the formation of a *trans*-SNARE complex, in which the vesicle is 'pinned' to the target membrane by the two sets of SNAREs. In step 3, membrane fusion occurs, the SNAREs are now on the same membrane in a *cis*-SNARE complex. Step 4 is the disassembly step, in which the SNAREs are separated and recycled for further rounds of fusion. In step 5 syntaxin reverts back to its 'closed' conformation (Carr and Novick, 2000). These steps are regulated by a large number of accessory factors. Important among these, are NSF (N-ethylmaleimide-sensitive fusion protein), and α-SNAP (soluble NSF attachment protein), which are involved in the disassembly of the *cis*-SNARE complex. Sec1 is thought to be part of the machinery that converts syntaxin 1 from its 'closed' conformation to its 'open' conformation (Misura *et al.*, 2000).

Accuracy in membrane fusion is vital for the growth and division of cells, and for maintaining the spatial organisation of biochemical events within the cell. The SNARE hypothesis proposed that v- and t- SNAREs provide the specificity for membrane docking and fusion (Rothman, 1994). This view was consistent with the observation that ER to Golgi transport in yeast can proceed in the absence of the yeast Ypt1 GTPase, if the v- and t-SNAREs involved in the pathway are overexpressed (Dascher *et al.,* 1991). This hypothesis has proved controversial ever since it was proposed. A body of work implicates the Rab GTPases and additional peripheral membrane proteins as providing the specificity for the docking events, and that the

SNAREs are simply present to drive the fusion event. Cao and colleagues used a yeast *in vitro* fusion system that measures the fusion of ER-derived vesicles to Golgi membranes. They demonstrate that Uso1p, a peripheral membrane protein, is required for docking and that removal of Ypt1p from the membranes with GDI inhibits this docking step. Fusion is not inhibited if GDI is added after docking has occurred. Using temperature sensitive SNARE mutants they show that this Ypt1p-regulated docking event is independent of SNARE function (Cao *et al.,* 1998). Other studies that analysed the interactions of purified SNARE proteins revealed that they were promiscuous in their interactions i.e. the authors observed interactions between SNAREs that could not realistically interact *in vivo* due to their subcellular localisation and participation in different transport pathways (Fasshauer *et al.,* 1999; Yang *et al.,* 1999). The conclusion was that since v- and t- SNAREs did not interact exclusively with their cognate partners they could not provide the specificity for the vesicle docking and fusion events.

Recent work from Rothman and colleagues has shifted the balance of results back in favour of the SNARE hypothesis. They used an *in vitro* assay in which purified yeast SNAREs were reconstituted in liposomes. This assay differs from previous assays in that interactions are measured between SNAREs that are in their natural membrane anchored conformation. The ability of all the known v-SNAREs in the yeast genome to trigger fusion by pairing with the t-SNAREs that label the plasma membrane, vacuole, and Golgi, was tested. Of the 33 possible combinations, only the four previously known cognate combinations and 2 new combinations had the ability to mediate fusion reactions (McNew *et al.,* 2000). Using this assay to analyse in detail the ER to Golgi fusion event, which involves the four SNAREs, Sed5, Bos1, Sec22, and Bet1, each of which contribute a single helix to the SNARE complex, it was demonstrated that only one combination in which the v-SNARE Bet1 is on one membrane, and the remaining three t-SNAREs are on the other, allows fusion to occur (Parlati *et al.,* 2000). Thus each SNARE is restricted by design to function as either a v-SNARE, or as part of a t-SNARE complex.

To conclude, the recently available data indicate that the SNARE hypothesis holds true. The specificity of intracellular membrane fusion appears to be mediated, to a large extent, by the SNARE proteins. However, even though a significant part of the specificity of vesicle transport is determined by the specificity inherent in cognate SNARE complexes, it is likely that this is complemented by a large number of

accessory proteins. These accessory proteins may be regulated by the activation state of specific Rab GTPases. The protein complexes recruited to vesicle membranes by activated Rabs are likely to have complementary, rather than redundant properties, with respect to the SNARE proteins. They are involved in the tethering of vesicles, their movement, and may permit the fine spatial and temporal control of fusion, by regulating the localisation and activation state of the SNAREs. Rothman *et al* propose that SNAREs provide the core, evolutionarily conserved, mediators of membrane compartment specificity, while Rab GTPases provide the fine-tuning for this specificity.

1.3 The Rab Family of Small GTPases

1.3.1 The Evolution of Rab Family

Rab GTPases ('Ras-related in brain'), and their yeast counterparts, Ypt proteins, form the largest branch of the Ras superfamily of small GTPases. They are evolutionarily conserved and are present in all eukaryotes investigated. Yeast has 11 members, while *C. elegans* and *D. melanogaster* have 29 and 26 members, respectively (Stenmark and Olkkonen, 2001). The human genome project has allowed the identification of at least 60 members in the human Rab subfamily (Bock *et al.,* 2001). Most Rab proteins are ubiquitously expressed, however, a number such as Rab3A, which has been implicated in secretion in neuroendocrine cells, are cell-type specific. Ten Rab subfamilies have been identified, the members of which appear to be the result of gene duplications and are between 75-95% identical (Figure 3). Members of these subfamilies have similar subcellular localisation and some functional overlap. Rab GTPases share their highest sequence conservation at their nucleotide binding domains, and are most divergent at their carboxy-termini, which are involved in their subcellular targeting (Chavrier *et al.,* 1991). Crystallographic studies have identified two regions, switch I and switch II, which undergo extensive conformational changes depending on the nucleotide-bound state of the Rab GTPase. These switch regions are important for binding to regulatory proteins such as GAPs and GEFs.

Resolution of the crystal structure of Rab3a, bound to its effector Rabphilin-3a, indicates that the switches form a major part of the interaction interface between the two proteins (Ostermeier and Brunger, 1999). Bioinformatic analysis of the amino acid sequences of the Rab family has identified four regions, close to the switch domains, that can be used to distinguish between subfamilies. These RabSF regions are present on two different surfaces of the protein and are proposed to allow the specific binding of downstream effector proteins (Moore *et al.,* 1995). It has been proposed that regulatory proteins and downstream effectors bind to the switch regions, to determine the nucleotide-bound state of the GTPase, and to the RabSF domain, to determine the subfamily specificity.

Rab GTPases localise to the cytoplasmic face of intracellular compartments where they regulate specific transport events (Figure 4). When in their GTP-bound, active, conformation they can recruit effector molecules. It has been difficult to identify novel Rab effectors by bioinformatic approaches, as they tend to be very divergent. There are potentially many more Rab effectors than Rab GTPases, since several Rabs have been shown to have more than one effector. The GTP-bound form of Rab5 has been shown to interact specifically with at least 20 different proteins (Christoforidis *et al.,* 1999a). Some effectors have coiled-coils and are involved in vesicle tethering and fusion, while some are enzymes, or cytoskeletal motor proteins. Identification of Rab effectors, and regulatory proteins, is a very important part in understanding the function of Rab GTPases.

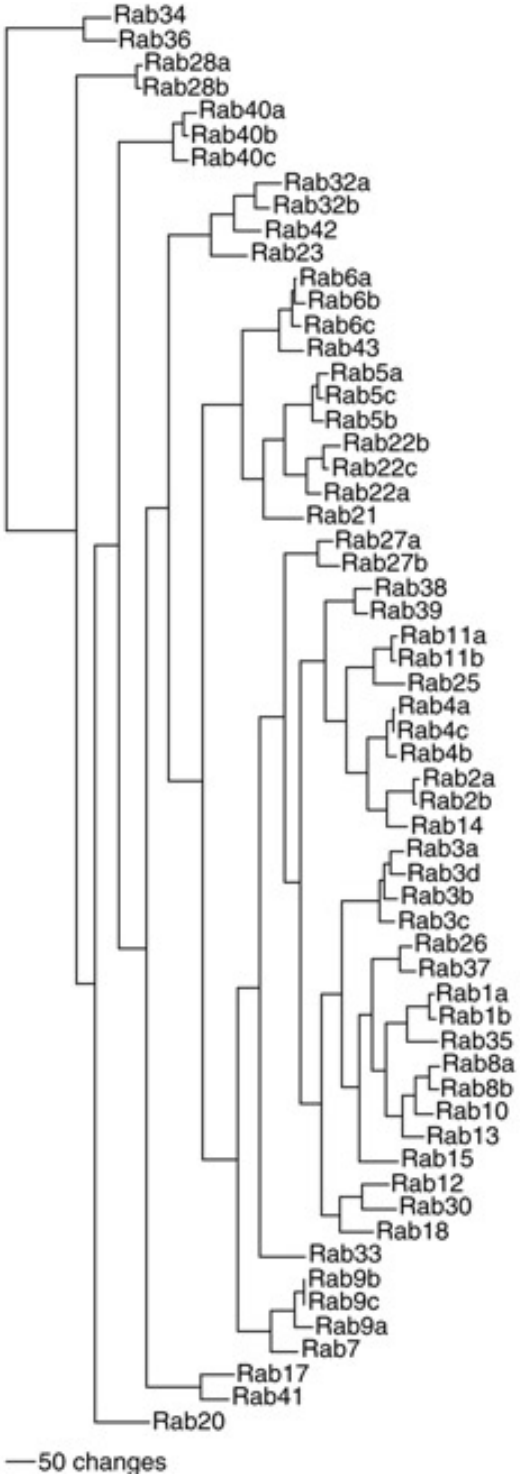

Figure 3. Phylogenetic tree of the human Rab GTPase family (Bock *et al.*, 2001).

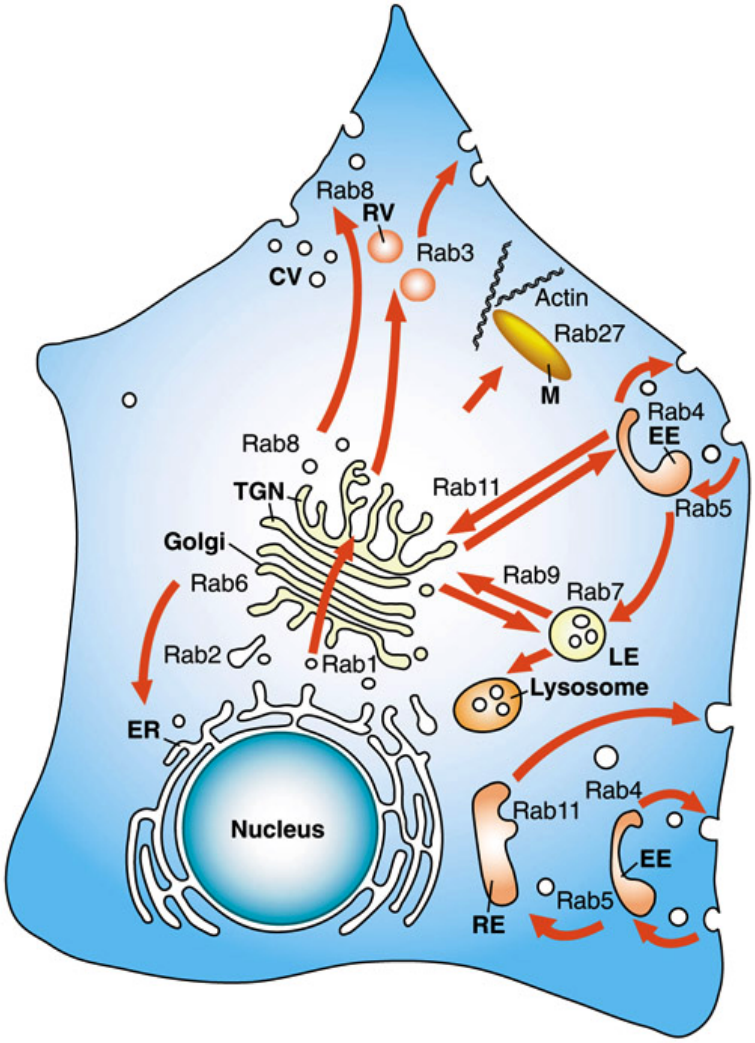

Figure 4. Intracellular transport pathways regulated by Rab GTPases (Stenmark and Olkkonen, 2001).

1.3.2 The Rab Cycle

Rab proteins, in common with the other GTPases of the Ras superfamily, cycle between an active and an inactive conformation. They are in their active, membrane-bound, state when bound by GTP. Hydrolysis of the GTP to GDP results in the conversion to their inactive conformation. Various accessory factors regulate this cycle. The current view of the Rab cycle proposes that GDP-bound Rab is maintained in the cytosol by Rab GDI (GDP-dissociation inhibitor), of which there are several isoforms in mammalian cells. Rab GDI has the ability to extract GDP-

bound Rab from membranes, but it can also deliver the Rab-GDP to its target membrane from the cytosol. Following delivery of the Rab to its specific compartment, a factor displaces Rab GDI enabling Rab-GDP to bind to the membrane. This may be mediated by a unique receptor on the target membrane (Ayad *et al.,* 1997). Next, the GDP is replaced with GTP by an exchange factor, or Rab GEF (guanine nucleotide exchange factor). GTP-bound Rab is now resistant to extraction by Rab GDI. Exchange factors can be highly specific, such as Rab3-GEF, or can have a broad substrate specificity, such as Mss4 (Burton *et al.,* 1994). These factors are very important in the transport process as they ensure the correct targeting and activation of the Rab GTPase. The GTP-bound Rab is now able to perform its regulatory function, usually as part of a large complex of effector proteins. Rab GTPase-activating proteins (Rab GAPs) stimulate the intrinsic GTPase activity of the Rab protein resulting in the conversion of the GTP to GDP, allowing Rab GDI access to the membrane-bound Rab, and its extraction from the membrane (Figure 5). The cycle starts over again;

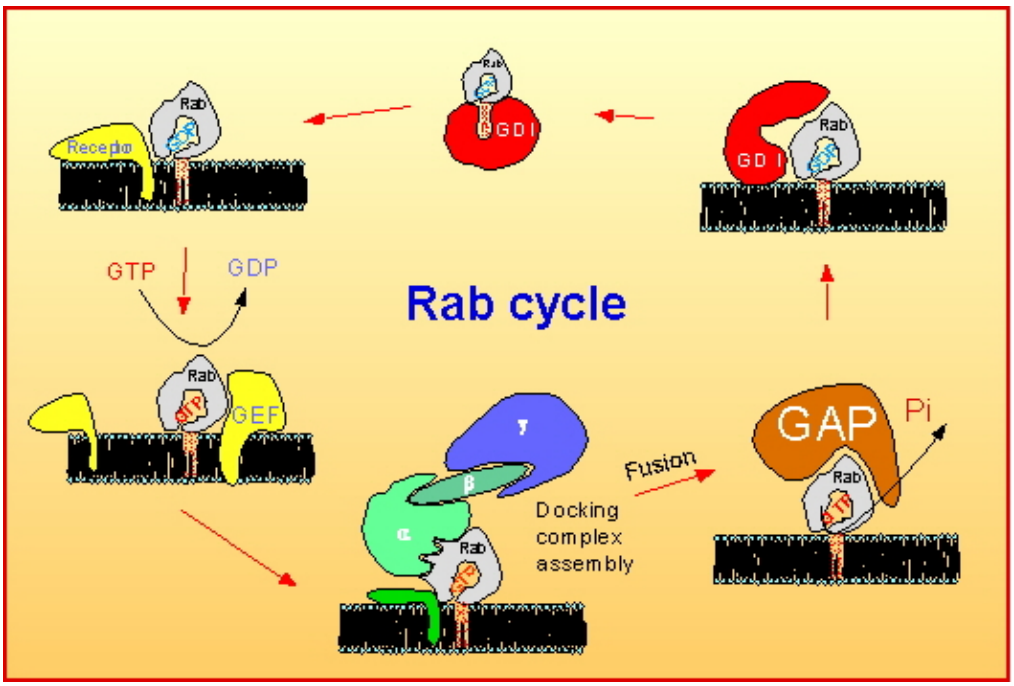

Figure 5. Overview of the Rab cycle (**www.mpi-dortmund.mpg.de/departments /dep3/alexandrov/kirillfinal/rab_cycle.html**).

In order for Rab proteins to bind membranes they must possess one, or two, C20 geranylgeranyl lipid groups at their carboxyl-terminus. The process by which they gain these lipid moieties is known as prenylation. Newly synthesised, unprenylated, Rab GTPases are bound by REP (Rab escort protein) and delivered to geranylgeranyl transferase (GGTase). GGTase is an enzyme that catalyses the attachment of the lipid groups to two carboxy-terminal cysteine residues present on all Rab GTPases (Anant *et al.,* 1998). REP shares a strong homology with Rab GDI and can itself deliver Rab proteins to membranes following geranylgeranylation. However, none of the Rab GDIs can replace REP. REP appears to be a specialised Rab GDI that can deliver newly synthesised, GDP-bound, Rab proteins to GGTase for isoprenylation, and then transport the modified Rab protein to its target membrane. Rab GDI then takes over in the subsequent cycling between cytosol and membrane.

1.3.3 The Role of Rab5

Rab5 is one of the most characterised Rab GTPases. It localises predominantly to the sorting endosome, but is also found at the plasma membrane. It has been implicated in the budding of vesicles from the plasma membrane, regulating endosomal membrane fusion, and cytoskeletal transport. Overexpression of wild-type, or constitutively active Rab5, Rab5Q79L, leads to an increase in the rate of ligand internalisation from the plasma membrane, and the formation of abnormally large early endosomes (Bucci *et al.,* 1992; Gorvel *et al.,* 1991). Rab5-GTP was shown to promote the homotypic fusion of sorting endosomes, and the heterotypic fusion of plasma membrane derived clathrin coated vesicles (CCVs) with early sorting endosomes. It is postulated that these activities are regulated by the recruitment of a scaffold of cytosolic proteins. Rab5-GTP has been demonstrated to interact specifically with at least 20 different cytosolic proteins (Christoforidis *et al.,* 1999a; Christoforidis *et al.,* 1999b). These include Rabaptin-5 and Rabex-5. Rabaptin-5 is a 100kDa protein that forms a homodimer and interacts with Rab5 at its C-terminus (Stenmark *et al.,* 1995). Rab5-GTP recruits Rabaptin-5 and Rabex-5, a Rab5-specific guanine nucleotide exchange factor, to the sorting endosomes. These proteins downregulate the intrinsic Rab5 GTPase activity thus providing a positive-feedback

loop that stabilises Rab5 on the membrane, and may generate areas of localised Rab5 accumulation (Horiuchi *et al.,* 1997; Lippe *et al.,* 2001).

Another homodimer, and Rab5 effector, is early endosomal antigen1 (EEA1), which is also essential for endosome fusion (Christoforidis *et al.,* 1999a; Mu *et al.,* 1995; Stenmark *et al.,* 1996). EEA1 is found on early endosomes and is thought to be involved in the docking process. It provides the directionality in the docking of CCVs with early endosomes, since Rab5 and Rabaptin-5 are found on both sets of membranes whereas EEA1 is recruited selectively to early endosomes (Rubino *et al.,* 2000). Its binding to early endosomes is mediated via a phosphatidylinositol-3-phopspahte [PI(3)P] lipid-binding FYVE domain (Stenmark *et al.,* 1996). EEA1 has the ability to bind the SNARE protein, syntaxin 13, thus providing a link between the SNARE complex and the Rab5 complex (McBride *et al.,* 1999).

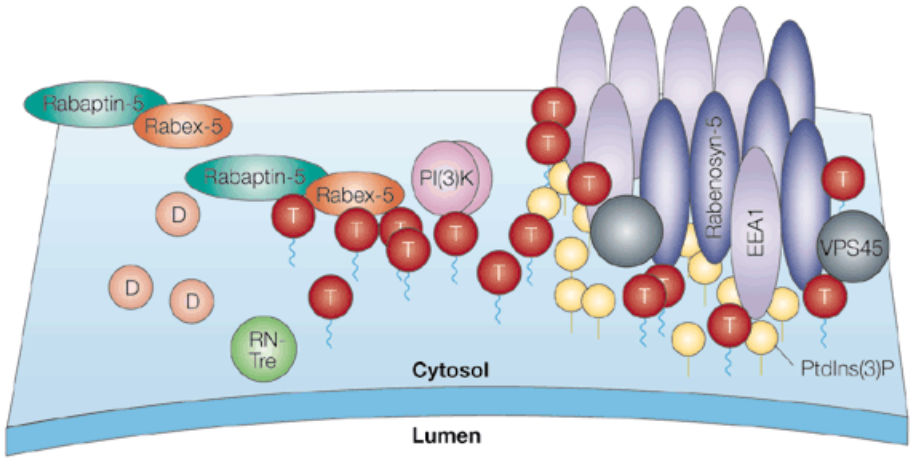

Figure 6. Rab5 recruits a scaffold of effector and regulator proteins to the cytoplasmic face of the early endosome (Zerial and McBride, 2001). Rab5-GTP, denoted as T is bound to the membrane, while Rab5-GDP, represented as D, is free in the cytoplasm.

Rab5 is also involved in the recruitment of cargo into clathrin-coated pits (McLauchlan *et al.,* 1998), and in the regulation of endosome motility via the recruitment, or activation, of a minus-end directed motor protein (Nielsen *et al.,* 1999). Thus a picture emerges of Rab5 regulating most aspects of the plasma membrane to early sorting endosome transport pathway, from the formation of clathrin coated pits, to the transport of CCVs to the early sorting endosome, and

finally to the docking and fusion of the CCVs with the sorting endosome. These diverse functions are regulated by the selective recruitment, by Rab5-GTP, of an array of proteins to specific locations along this pathway. There is increasing evidence that other Rab GTPases may also have similar 'universal' roles (see below).

1.3.4 Rab4 Regulates Transport from the Sorting Endosome

Rab4 is localised primarily to the early sorting endosome (Daro *et al.,* 1996; Van Der Sluijs *et al.,* 1991), although a minor pool is associated with the Rab11-positive recycling compartment (Sonnichsen *et al.,* 2000; Trischler *et al.,* 1999). Rab4 is involved in regulating transport along both the recycling and degradative pathways (McCaffrey *et al.,* 2001; van der Sluijs *et al.,* 1992). It is thought to regulate the recycling of receptors from the sorting endosomes either, directly back to the plasma membrane (Daro *et al.,* 1996), or via the pericentriolar recycling compartment (Mohrmann and van der Sluijs, 1999). Expression of a dominant negative mutant of Rab4, Rab4S22N, in mammalian cells results in an inhibition of both recycling and degradation. Overexpression of wild-type Rab4, or a dominant positive mutant, Rab4Q67L, causes the formation of a tubular network accessible to FITC-Tfn (McCaffrey *et al.,* 2001). During mitosis Rab4 is phosphorylated by $p34^{cdc2}$ kinase, which results in its translocation from the sorting endosomes to the cytoplasm, where it is retained by the peptidyl-prolyl isomerase, Pin1 (Barbieri *et al.,* 2000; Gerez *et al.,* 2000). This may be one mechanism by which the endocytic pathway is shut down during cell division.

Rab4 plays a regulatory role during the insulin-stimulated uptake of glucose in adipose and muscle cells. Insulin induces the translocation of a glucose transporter protein, GLUT4, from an intracellular store to the plasma membrane. Rab4 is present on GLUT4-containing vesicles and undergoes an insulin-induced redistribution from membranes to the cytosol. To determine whether Rab4 has a direct role in insulin-stimulated glucose transport, Shibata *et al* demonstrated that a peptide, corresponding to part of the carboxy-terminal hypervariable region of Rab4, inhibits GLUT4 translocation to the plasma membrane in permeablised rat adipocytes, as does the presence of anti-Rab4 antibodies (Shibata *et al.,* 1996). In contrast, peptides corresponding to the hypervariable-domains of Rab3C and Rab3D had little effect,

despite the fact that Rab3D is predominately expressed in this cell line. It was demonstrated that insulin stimulates the binding of GTP by Rab4, via a phosphoinositide 3-kinase dependent pathway (Shibata *et al.,* 1997).

A number of Rab4 interacting proteins have been identified. These include Rabaptin-5/Rabaptin-4 (Nagelkerken *et al.,* 2000; Stenmark *et al.,* 1995; Vitale *et al.,* 1998), syntaxin 4 (Li *et al.,* 2001), Rabip4 (Cormont *et al.,* 2001), and the light intermediate chain-1 of the motor protein dynein (Bielli *et al.,* 2001). Rabaptin-5 is a 100kDa protein that was identified in a yeast two-hybrid screen for Rab5 effectors. It was subsequently shown to interact with Rab4. The Rab interacting domains of Rabaptin-5 differ. Rab5 interacts with the carboxy-terminus of Rabaptin-5, whereas Rab4 binds to its amino-terminus. Rabaptin-5 is recruited from the cytosol, to the early sorting endosomes by GTP-bound Rab5. However, activated Rab4 does not appear to be able to recruit Rabaptin-5. It was postulated that Rab4 interacts with Rabaptin-5 that has already been recruited to the early endosome by Rab5. Since Rab5 and Rab4 cooperate in regulating the transport influx into, and efflux out of, early endosomes, Vitale *et al.* propose that Rabaptin-5 acts as a 'molecular bridge' that links these two sequential transport events (Vitale *et al.,* 1998).

Li *et al.* recently demonstrated that Rab4-GTP has the ability to bind syntaxin 4, a *t*-SNARE found at the plasma membrane (Li *et al.,* 2001). This interaction requires syntaxin 4 to be in the open conformation. The authors propose that syntaxin 4 is a target for Rab4-regulated vesicles, and provides a method by which Rab4 can direct the docking/fusion of transport vesicles with the plasma membrane. A yeast two-hybrid screen (our laboratory) identified the dynein light intermediate chain-1 as a Rab4-GTP interacting partner (Bielli *et al.,* 2001). Dynein is a motor protein that directs retrograde transport along microtubules. It is proposed that Rab4 directs the cytoskeletal transport of vesicles from the sorting endosome via its interaction with the dynein complex.

Rabip4 is a 69kDa, FYVE domain-containing, protein. It colocalises with EEA1 and Rab4 on the early sorting endosome, but does not colocalise with Rab11 at the recycling compartment. In common with Rabaptin-5 it has a number of coiled-coil domains. It is a membrane bound protein, cooverexpression of Rabip4 with Rab4Q67L leads to an enlargement of the sorting endosome and to an increase in the overlap between Rab5 and Rab4, and Rab4 with Rab11b. Expression of Rabip4 leads to the intracellular retention of the glucose transporter, GLUT1. It is proposed that

Rabip4 functions as the acceptor, on the sorting endosomes, for the pool of Rab4 that is regulating traffic back from the recycling compartment. From the available evidence it is emerging that Rab4 is a key regulator in the sorting process that occurs in the early sorting endosome, and that it regulates a number of different transport steps from this compartment. However, additional work is necessary to determine more precisely its role(s) in intracellular trafficking.

1.3.5 Rab11 Regulates Transport Through the Recycling Compartment

The Rab11 family consists of three members, Rab11a, Rab11b, and Rab25. It is a ubiquitously expressed protein, however gastric parietal cells appear to possess the highest endogenous levels of Rab11a. Rab11 is found on the perinuclear recycling compartment and the *trans*-Golgi network (TGN) (Ullrich *et al.,* 1996). It is involved in the regulation of transport from the recycling compartment, either back to the plasma membrane, or to the TGN (Urbe *et al.,* 1993). Overexpression of a dominant-negative mutant of Rab11, Rab11S25N, inhibits the recycling of Tfn from the recycling compartment (Ullrich *et al.,* 1996), and results in the formation of an extensive tubular FITC-Tfn positive network in HeLa cells (Wilcke *et al.,* 2000). This Rab11S25N-induced tubular network is inaccessible to EGF or Tfn internalised at 19^{o}C, suggesting that it is derived from the recycling compartment. Wilcke *et al.* demonstrate that Rab11 is also involved in transport from the endocytic compartment to the biosynthetic pathway by demonstrating that endogenous TGN38, a marker that cycles between the TGN and the plasma membrane, is redistributed into Tfn-positive structures in cells expressing the dominant-negative mutant. In polarised cells Rab11 is associated with the apical recycling system. Overexpression of Rab25 in polarised MDCK cells results in an inhibition of transcytosis, and apical recycling (Casanova *et al.,* 1999).

As with the other Rab GTPases discussed, a number of proteins have been shown to interact with Rab11. The first, Rabphilin-11/Rab11BP, was reported separately by two different groups (Mammoto *et al.,* 1999; Zeng *et al.,* 1999). Rabphilin-11 is an approximately 100kDa protein, the carboxy-terminus of which contains six WD40 domains. WD40 domains are approximately 42-44 amino acids in length and are thought to mediate protein-protein interactions. It is predominantly a

cytosolic protein but is recruited to the recycling compartment, and TGN, by Rab11-GTP. Overexpression of full-length Rabphilin-11 had no effect on Tfn recycling, however, overexpression of a truncated form that contained the Rab11 binding domain, resulted in a dramatic inhibition. The authors of both studies propose that Rabphilin-11/Rab11BP is a downstream effector of Rab11. In a yeast two-hybrid screen that used the amino-terminal fragment of Rabphilin-11 as bait, Mammoto *et al* identified mSec13p as a Rabphilin-11 interacting partner (Mammoto *et al.,* 2000). mSec13p is the mammalian homologue of yeast Sec13p which is involved, as part of the coat protein II (COPII) complex, in vesicle transport from the endoplasmic reticulum (ER). The role of mSec13p is unclear, but the authors show that it is localised to the ER and Golgi complex in BHK and MDCK cells. Rabphilin-11 and mSec13p colocalise in the perinuclear region, presumably the Golgi. Overexpression of active Rab11 enhances the Rabphilin-11/mSec13p interaction. The authors contend that the function of the Rab11/Rabphilin-11/mSec13p interactions in the cell are unclear, but hypothesise that one function could be in the budding of vesicles from the Golgi.

Rip11 is a 72kDa protein that interacts preferentially with Rab11-GTP. It was identified as a protein from a rat brain extract that bound to the GTPase-deficient, Rab11Q70L (Prekeris *et al.,* 2000). Microsequencing revealed that this protein matched the previously characterised KIAA0857 open reading frame. KIAA0857 had been described as a phosphoprotein, pp75, that was involved in the autoimmune condition lupus erythematosus (Wang *et al.,* 1999). It is characterised by a C2-phospholipid binding domain near its amino-terminus, and a coiled-coil region at its carboxy-terminus that mediates the interaction with Rab11. It is a predominantly membrane bound protein and Prekeris *et al.* localised Rip11 to the apical recycling compartment in polarised MDCK and Caco-2 cells. Overexpression of a truncation mutant, that contained the Rab11 binding domain, did not inhibit the trafficking of [^{125}I] Tfn, which was not surprising since Tfn is recycled from the basolateral surface back to the basolateral plasma membrane in polarised cells. However, there was a significant inhibition of [^{125}I] IgA transcytosis from the basolateral to the apical membrane. Analysis of the Rip11 primary sequence identified a number of putative phosphorylation sites. Treatment of Caco-2 cells with kinase and phosphatase inhibitors confirmed that the membrane binding of Rip11 is regulated by its phosphorylation state.

Prekeris *et al.* propose the following model for Rip11 function. Rip11 is recruited to the apical recycling compartment by Rab11-GTP, where it binds to neutral phospholipids via its C2 domain. The Rip11/Rab11-containing transport vesicle buds from this compartment and is transported to the apical plasma membrane, where Rip11 binds to an as yet unidentified receptor. After vesicle docking, a SNARE scaffold can form and mediate membrane fusion. Hydrolysis of GTP by Rab11 releases Rip11, and phosphorylation of Rip11 leads to its transfer to the cytosol from which it can be recruited back to the recycling compartment.

MyosinVb, a member of the class V family of unconventional myosins, was identified as a Rab11 interacting protein in a yeast two-hybrid screen of a rabbit parietal cDNA library (Lapierre *et al.,* 2001). Little is known about myosin Va or myosin Vb. Lapierre *et al* demonstrate that myosin Vb colocalises with Rab11 in the perinuclear region of HeLa and MDCK cells. No colocalisation was seen with Golgi markers. Overexpression of its carboxy-terminal 60kDa, which contains the Rab11 binding sites but lacks the motor domain, in HeLa cells, results in an inhibition of the traffic of transferrin from the recycling compartment to the plasma membrane. Expression of this construct in MDCK cells caused an inhibition in the transcytosis of IgA, but did not affect the trafficking of Tfn internalised from the basolateral membrane. The authors propose that myosin Vb is involved in Rab11-regulated trafficking out of the recycling compartment. Myosin Vb can now join the list of motor proteins that interact directly with, or are regulated by Rab GTPases. It is increasingly likely that each Rab protein may interact with a motor protein.

1.3.6 Cross-talk Between Rab GTPases and Signal Transduction Pathways

Evidence is emerging that a number of different signalling pathways may mediate their effects on endocytosis via “cross-talk” with Rab GTPases. Such pathways include the stress response pathway and the EGFR-signalling pathway. Rac is a member of the Rho family of small GTPases (Rho GTPases function largely to regulate the actin cytoskeleton in response to signals from cell surface receptors), and is activated by the EGF receptor. The activation of Rac is mediated by Eps8, a substrate of the EGFR, in complex with Sos1 and the adaptor protein, E3b1. RN-tre

was identified as an Eps8 interacting protein. Recent work has demonstrated that RN-tre is a Rab5-GAP, i.e. stimulates the GTPase activity of Rab5 (Lanzetti *et al.,* 2000) (see Figure 6). Thus, it was believed that RN-tre might provide a link between EGFR signalling and its endocytosis. Unlike the TfnR, which is constitutively endocytosed, the EGFR is internalised only upon activation by EGF. The authors demonstrated that RN-tre overexpression inhibits EGFR and TfnR internalisation. This inhibition was expected, since a Rab5-GAP would promote the conversion of active Rab5 to its GDP-bound, inactive form. To demonstrate a link between Rab5 and Rac signalling, RN-tre was overexpressed in Eps8 null fibroblasts. In these cells, trafficking of the TfnR was still inhibited, however internalisation of the EGFR was unaffected. Thus, the interaction between Eps8 and RN-tre is necessary for inhibiting Rab5 mediated ligand-dependent endocytosis. The authors propose that the RN-tre/Eps8 complex bound to the EGFR, excludes E3b1 from binding Eps8 and activating Rac. RN-tre localised to the EGFR inhibits Rab5-mediated endocytosis of the receptor. The binding of ligand to the EGFR results in the dissociation of RN-tre from Eps8, thus facilitating the binding of E3b1/Sos1 and the subsequent activation of Rac. The absence of RN-tre removes the block on EGFR internalisation and allows the downregulation of its signal.

Guanine-dinucleotide dissociation inhibitor (GDI) is phosphorylated in the cytosol, suggesting that a kinase can regulate the membrane association of Rab GTPases. Cavalli *et al.* recently investigated the mechanism of this regulation by analysing the ability of GDI to extract Rab5 from early endosomes (Cavalli *et al.,* 2001). Using an assay in which purified GDI was used to extract Rab5 from early endosomes, the authors determined that GDI was activated by an enzyme present in the cytosol, and that this enzyme was probably a kinase since activation was inhibited by the presence of nonhydrolysable analogues of ATP. This factor was identified as the protein kinase p38 MAPK. H_2O_2 is an activator of p38 MAPK in the p38-dependent stress response. It was shown that treatment with H_2O_2 resulted in increased GDI phosphorylation, and in the amounts of GDI:Rab5 complex *in vivo*. Specific inhibitors of p38 MAPK reduce the ability of GDI to extract Rab5. Immunofluorescence studies revealed that H_2O_2 causes a partial redistribution of the Rab5 effector EEA1, from membranes to the cytosol. H_2O_2 also stimulates the early stages of endocytosis. The authors propose the following model to explain this stimulation of endocytosis upon activation of p38 MAPK; they suggest that the

amount of the cytosolic GDI:Rab5 complex is rate-limiting at each round of transport [approximately 5-10% of Rab5 is cytosolic, the rest being membrane-bound. Recent studies have shown that only 10-20% of the membrane-associated pool of Rab proteins is sufficient to sustain growth in yeast]. The oxidative stress induced activation of p38 MAPK, activates GDI which extracts Rab5 that is undergoing futile GTPase cycles on the membrane, and delivers it to the plasma membrane where it can mediate the formation of endocytic vesicles. Alternatively, the Rab5 may be delivered to active regions of the membrane where it will regulate docking/fusion, or interactions with the cytoskeleton.

A number of other Rab interacting proteins have been identified whose membrane-association appears to be mediated by phosphorylation, and are thus potential targets for regulation by external stimuli. The membrane binding of Rip11 is regulated by phosphorylation (Prekeris *et al.,* 2000). Rab proteins themselves can be phosphorylated. Rab4 is phosphorylated upon activation of ERK1 kinase by insulin, which results in its redistribution into the cytosol (Cormont *et al.,* 1994). It can be speculated that external stimuli contribute to the regulation of intracellular trafficking for the purposes of promoting repair (e.g. the stress-response), storage (e.g. the insulin-stimulated translocation of GLUT4 to the plasma membrane for the purpose of glucose uptake), and degradation (e.g. the downregulation of the EGF signal by transporting the EGFR to the lysosomes).

1.3.7 The Involvement of Rab GTPases in Disease

The completion of the human genome sequencing project has allowed the identification and functional characterisation of a large number of disease causing genes. It is becoming apparent that many of these mutated genes cause defects in intracellular trafficking (Aridor and Hannan, 2000). Several of these genetic diseases are the result of mutations in Rab GTPases, or their interacting proteins (Stenmark and Olkkonen, 2001). Griscelli syndrome is an autosomal recessive disorder that results in pigmentation defects. There are two variants of this disease, one that results in immunological defects, and the other is associated with neurological dysfunction. The former is caused by missense mutations in the Rab27a gene, and the latter is the result of mutations in myosin Va. Both are located side-by-side on chromosome 15,

and mysoin Va is a putative effector of Rab27a. Rab27a is involved in regulating the transport of melanosomes to the periphery of melanocytes, and it also regulates the secretion of lytic granules in cytotoxic T lymphocytes. Hence, the lack of functional Rab27a results in the loss of pigmentation and in the uncontrolled activation of T-lymphocytes. Myosin Va is not involved in the exocytosis of lytic granules, hence its inactivation does not cause the immunological defects observed upon inactivation of Rab27a (Stenmark and Gillooly, 2001).

Tuberin is a tumour suppressor gene that interacts with Rabaptin-5 and negatively regulates endocytosis by acting as a Rab5-GAP. Its inactivation causes tuberous sclerosis, a disease that results in malformations and tumours on the central nervous system (Xiao *et al.,* 1997). Choroideremia is an X-linked disease that results in the degeneration of the pigment epithelium of the eye. It is caused by a defect in the REP-1 gene. REP-1 is one of the two isoforms of gernayl-geranyl transferase that prenylate Rab GTPases. REP-1 is essential for the prenylation of Rab27a in the retinal pigment epithelium. REP-2 appears to be sufficient for the efficient geranyl-geranylation of all other Rab GTPases, in all other tissues. Thus, the lack of functional REP-1 results in non-functional Rab27a and the onset of the disease. The pathology of this disease may be caused by the degeneration of the retinal epithelium due to deficient melanosome transport, and hence lack of protection during light exposure (Stenmark and Olkkonen, 2001). Mutations in the GDI1 gene, which codes for GDI-α, have been found in a subgroup of patients with X-linked non-specific mental retardation. GDI-α is particularly abundant in brain tissue, thus defective membrane recycling of one or more Rab GTPases in brain synapses is likely to be the cause of this condition.

2 Materials and Methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich Ltd. All restriction enzymes and Taq polymerases were from Roche Diagnostics or New England Biolabs, unless otherwise stated. All bacterial media was purchased from Difco, and yeast defined media was from Bio101. The bacteria and yeast strains used are listed below.

E.coli bacterial strains used, included;

HB101 *thi*-1, *hsd* S20, (r_B^-,m_B^-), *sup*E44, *rec*A13, *ara*-14, *leuB*6, *proA*2, *lac*Y1, *rpsL*20(str^r), *xyl*-5, *mt*1-1

BL21(DE3)pLysS F$^-$, *omp*T, *hsd*S_B, (r_B^-,m_B^-), *dcm, gal,* λ(DE3), pLysS(Cm^r)

XL-1 Blue *rec*A1, *end*A1, *gyr*A96, *thi-1, hsd*R17, *sup*E44, *rel*A1, *lac* [F' *pro*AB, *lacI*^{q}, ZδM15, Tn10, (Tet^r)]

The strain of *Saccharomyces cerevisiae* used for all yeast two-hybrid work was;

L40 *MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-lacZ*

2.2 Yeast Two-Hybrid Methods

2.2.1 Screening of an oligo-dT Primed HeLa cDNA Library

S.cerevisiae L40 was transformed with the 'bait' vector, pVJL10 Rab4Q67L (-CXC). A fresh colony from the transformant plate was used to inoculate a DO W$^-$ (media lacking tryptophan) liquid culture. After growth overnight, the medium was diluted to an OD_{600}=0.5 and allowed to grow for a further 2 hours. The cells were harvested and resuspended in 100mls of YPD (1% yeast extract, 2% peptone, 2% D-glucose) and grown to an OD_{600}=1.1. The cells were harvested as before, washed in 0.1M lithium acetate/TE, and finally resuspended in 4ml 0.1MLiAc/TE. Fifty micrograms of the oligo-dT primed HeLa cDNA library (MATCHMAKER, Clontech) was mixed with denatured salmon testis DNA and added to the cell suspension. Twenty-eight millilitres of 50% polyethylene glycol/0.1M LiAcTE was added, and the mixture was incubated for 30 minutes at 30^{o}C. After this incubation, 3.5ml DMSO was added and the cells were heat-shocked at 42^{o}C for 20 minutes. The cells were pelleted, washed once in DO W$^-$L$^-$ (media lacking tryptophan and leucine) liquid medium, and resuspended in 100ml of the same medium. This culture was

grown for 8 hours, harvested, and plated on DO $W^-L^-H^-$ (media lacking tryptophan, leucine, and histidine) agar plates. Colonies that appeared after 3 to 5 days of incubation were patched onto DO W^-L^- plates for further analysis.

2.2.2 Yeast Transformations

One hundred millilitres of fresh YPD was inoculated with a 10ml overnight culture of L40. The culture was incubated in a 30°C shaking incubator for approximately 2 hours until it reached a titre of 1-3x10^7 cells/ml. The cells were then pelleted at 1000g for 5 minutes, and the pellet was washed with 45ml 0.1M lithium actetate/TE, then resuspended in 2mls 0.1M lithium acetate/TE and incubated for 1 hour at 30°C. Meanwhile, plasmid mixtures were prepared. 1-2µg each, of the 'bait' and 'prey' plasmids were mixed with 4µl of denatured salmon testes DNA (10mg/ml). 150µl of the L40 in 0.1M lithium acetate/TE was added to the plasmid mixture and incuabted at 30°C for 10 minutes, followed by the addition of 500µl of 50% polyethylene glycol (PEG, M.W. 3350) in 0.1M lithium acetate/TE. Incubation at 30°C, with occasional mixing, was continued for 1 hour. The yeast was then heat shocked at 42°C for 25 minutes. The transformations were spun for 5 seconds at top-speed in a bench-top centrifuge. The pellets were washed twice with distilled water and resuspended in 200µl dH_2O. The resuspended cells were plated on selective media, lacking leucine and tryptophan (DO W^-L^-), and incubated for 3 days in a 30°C plate incubator.

2.2.3 *Rescue of Library Plasmids from S.cerevisiae* L40

Single colonies were picked and grown overnight at 30°C in 100ml DO L^- medium. The cells were pelleted and resuspended in 5ml yeast lysis buffer (2% Triton X-100, 1% SDS, 1M NaCl, in TE). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and 2g glass beads (0.45mm diameter, Sigma) were added and the solution was vortexed for 15 minutes. The debris was pelleted at 3000g for 5 minutes. The DNA-containing, aqueous phase, was transferred to a fresh tube and ethanol precipitated. The DNA pellet was resuspended in 10µl of dH_2O and used to transform

E.coli HB101 cells. Several colonies were picked and a quick screen method was used to identify library plasmids, which were approximately 10kb in size compared with the bait plasmid of 5.6kb.

2.2.4 β-galactosidase Filter Assay

Clones were patched onto DO W^-L^- plates and grown overnight at 30°C. A sterile Whatman 40 filter was placed on a fresh W^-L^- plate, onto which the patches were replica plated. The patches were grown on the filter overnight at 30°C. Once the patches had grown the filter was lifted from the plate and snap-frozen in liquid nitrogen. After thawing, the filter was soaked in Z Buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7) containing 6.75µl β-ME and 25µl 2% X-Gal, and placed in a 30°C incubator. A blue colour was allowed to develop. The reaction was stopped after 2 hours by soaking the filter in 1M Na_2CO_3.

2.2.5 β-galactosidase Liquid Assay

Five millilitres of DO W^-L^- liquid media was inoculated with several colonies from a freshly grown transformation plate and grown overnight at 30°C, with shaking. The overnight culture was titred (OD_{600} 0.1=~$1x10^6$ cells/ml) and subcultured into 10ml of fresh media, to a titre of $5x10^6$ cells/ml. This was incubated for approximately two hours until the titre was 1-$2x10^7$ cells/ml. The OD_{600} of this culture was taken by diluting 500µl of the culture into 500µl of dH_20. Three millilitres of the culture was pelleted and resuspended in 100µl of Z Buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 1mM KC1, 0.1mM $MgSO_4$). The resuspended pellet was snap-frozen in liquid nitrogen three times, after which 700µl of Z Buffer with 0.03% β-mercaptoethanol (β-ME) was added. 160µl of a freshly made solution of ONPG (4mg/ml), in Z Buffer, was added and the reaction was allowed to proceed, at 37°C, until a yellow colour developed. The reaction was stopped immediately with 400µl 1M Na_2CO_3. The start and finish time was noted. Cell debris was pelleted at 13,000g for 5 minutes. The OD_{420} of the supernatant was measured. The units of βgalactosidase activity were calculated using the following equation;

$$\text{Units}=(OD_{420}x1000)/(t \times V \times OD_{600})$$

't' is the elapsed time (in minutes), V is the volume of culture used in millilitres, and OD_{600} is the optical density of the culture.

Note: Most incubation times for reactions containing two interacting hybrid proteins were usually no longer than five minutes. All reactions that showed no colour after 30 minutes were stopped at that point.

2.3 Molecular Biology

Subcloning, bacterial transformations, agarose gel electorphoresis, and purification of DNA bands from agarose gels, were performed using standard molecular biology techniques, or according to the protocol supplied by the manufacturer of the reagent or kit used. All cDNA fragments that were amplified by PCR were verified by sequencing (Dept. Biochemistry, Oxford University).

2.3.1 Plasmids

The vectors, pcDNA3.1HisB H13, and pTrcHisC H13, were constructed by ligating the 1kb EcoRI fragment from pGADGH H13 into the EcoRI sites of both vectors. δRCP was amplified from a placental cDNA library using Vent DNA polymerase (NEB) and the primers δRCP Fwd and GSP6. The primers contained BamHI restriction sites at their 5'ends. The product was cleaned up using the Geneclean II kit (BIO101) and digested with BamHI and ligated 'in-frame' into the BamHI sites of pGADGH, pGEX6P-1, and pcDNA3.1HisB. Full-length RCP was encoded in IMAGE Clone 3956619. The approximately 2.2kb from EST 3956619 was subcloned into the EcoRI site of the pGEM1 vector, and hence put under the control of the T7 promoter. Rab4WT was digested from pTrcHisA and subcloned into the BamHI site of the GST-expression vector, pGEX2T. The Rab11 effector mutant was constructed by two-step site directed mutagenesis, using a mutagenic primer that introduced a glutamate codon for the isoleucine at amino acid position 44, and an

upstream vector primer. The product of this reaction was used as the upstream primer in the second step in conjunction with a downstream vector primer.

KIAA0941 was requested from the Kazusa DNA Databank, where it had been cloned into the pBluescript SK II vector. The open-reading frame was amplified using Expand DNA polymerase (Roche) with the KIAA0941Fwd(+EcoRI) and KIAA0941Rev(+EcoRI) primers and the Kazusa construct as template. The A-tailed product was ligated into the pGEM-T vector (Promega), from which it was digested with EcoRI and subcloned into the EcoRI sites of pGADGH, pcDNA3.1HisB, and pEGFP-C1. The GFP-0941CT vector was constructed by digesting the parent vector with PstI, and ligating the resulting fragment into the PstI site of pEGFP-C1. The backbone was purified and religated to give the GFP-0941NT vector. The KIAA0941CT 6XHis-fusion vector was constructed by ligating the HindIII fragment, coding for amino acids 276-511, into the HindIII site of pTrcHisA. The backbone from this digestion was relegated to produce pTrcHisA KIAA0941NT (codes amino acids 1-275). The EcoRI/SalI fragment from this vector was subcloned into the same sites in pGADGH to give pGADGH KIAA0941NT, and the EcoRI fragment from pEGFP-C1 KIAA0941CT was subcloned into the EcoRI site of pGADGH to give pGADGH KIAA0941CT.

Primer	Primer Sequence	Tm
δRCPFwd	5'-CG**GGATCC**CGCAGAAGACGCATGGTATAAG-3'	60
GSP6	5'-CG**GGATCC**GCCCTGGATCTAAGCAGAAGGTATT-3'	63
KIAA0941Fwd	5'-CG**GAATTC**CCTGTCCGAGCAAGCCCAAAAG-3'	58
KIAA0941Rev	5'-CG**GAATTC**GAAACCAGCCACAGGATCAATTC-3'	56
Rab11I44E	5'-TGCAAACTCTACTCCCTCGGTGCTCTT-3'	63

Table 1. Primer sequences. Introduced restriction sites indicated in bold. Oligonucleotides were supplied by Genset.

2.3.2 Plasmid Quickscreen

25μl of overnight *E.coli* cultures were centrifuged at 9000rpm in a benchtop centrifuge (Eppendorf) and resuspended in 50μl of 1X Cracking Buffer (700μl 2X

Cracking Buffer, 420µl dH_2O, 140µl 6X DNA loading dye, 140µl glycerol; 2X Cracking buffer contains 100mM NaOH, 10mM EDTA pH 8.0, 1% SDS, 10% glycerol) and incubated at room temperature for 5 minutes. 15µl was then separated on a 0.8% agarose gel.

2.3.3 Rapid Amplification of cDNA Ends (RACE)

Each 5' RACE reaction used 3µl of an adaptor-ligated placental cDNA library as template, the adaptor primer (AP1, 50pmol), gene-specific primer 3 (GSP3, 50pmol), 0.2mM dNTPs, 10µl 10X Vent Buffer, and 1 unit Vent DNA polymerase (New England Biolabs), in a total volume of 100µl. The PCR parameters were forty cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. As no specific bands were seen, 1µl of this reaction was used as template for a nested PCR, which used adaptor primer 2 (AP2, 50pmol) and Nested-GSP3 (N-GSP3) as an internal gene-specific primer;

Primer	Primer Sequence	Tm
AP1	5'-CCATCCTAATACGACTCACTATAGGC-3'	70
AP2	5'-ACTCACTATAGGGCTCGAGCGGC-3'	70
GSP3	5'-TTGAAGTCGGCAGGACAGACATGGACTGGGAAAGAGG-3'	76
NGSP3	5'-GCGGTGTCTGACCCACTGTCCTTATTCTTC-3'	66

.

Table 2. Primers used for the 5' RACE reactions.

The same cycling parameters were used for the nested PCR. The products of this reaction were cleaned up using the Geneclean II kit (BIO 101), and A-tailed with 200µM of dATP and 1 unit of Taq, at 72°C for 20 minutes, allowing subsequent ligation into a T-vector (pGEM T-Easy, Promega).

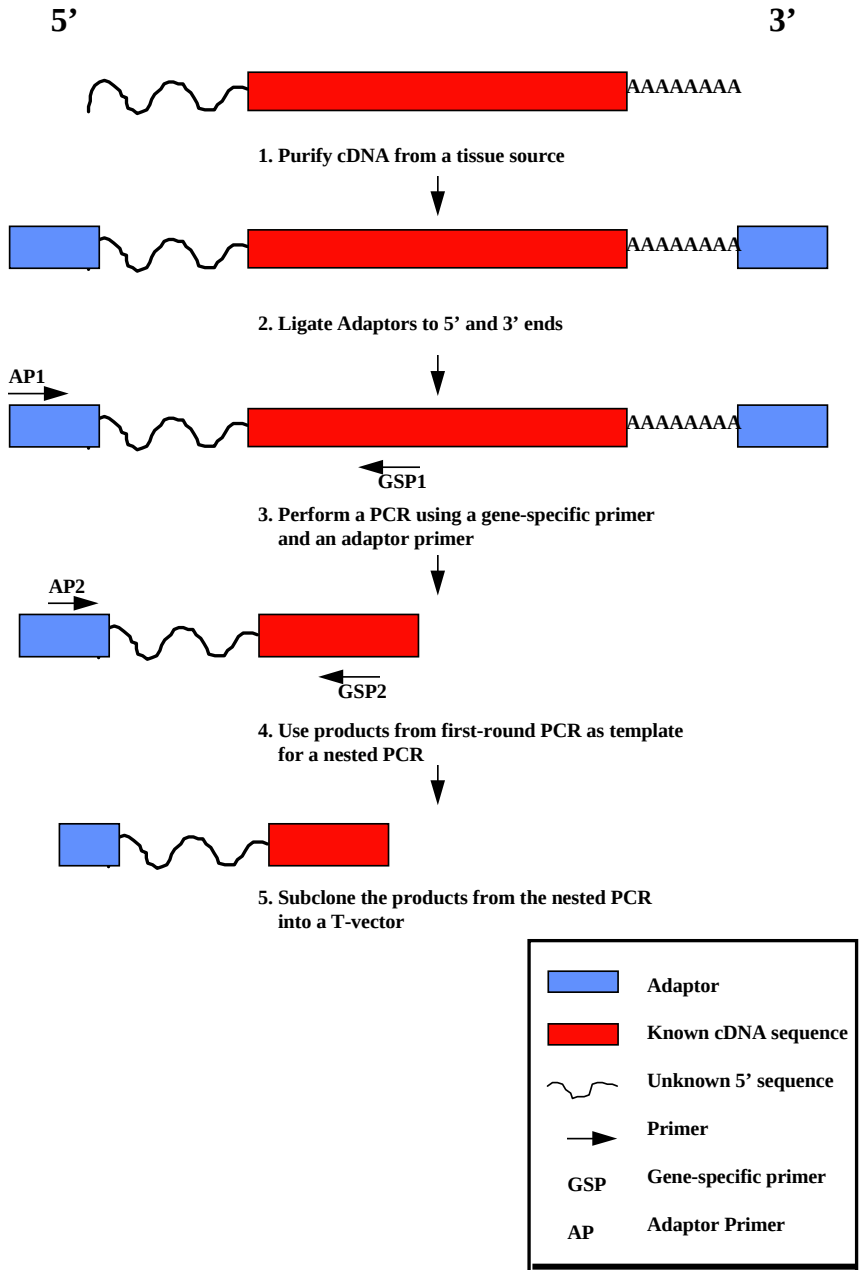

Figure 7. Overview of 5’-directed RACE procedure

2.4 Protein Purification and Production of Antiserum

SDS-polacrylamide gel electrophoresis (PAGE), Western blotting, and Coomassie Blue gel staining, were performed using standard techniques.

2.4.1 Protein Purification

2.4.1.1 Purification of 6XHis-tagged Proteins

Ten millilitres of LB (1% NaCl, 1% tryptone, 0.5% yeast extract, pH 7) containing ampicillin (50μg/ml) and chloramphenicol (34μg/ml) was innoculated with *E.coli* BL21, containing the appropriate 6XHis-tagged construct, and grown overnight at 37^{o}C. This was used to inoculate 1 litre of LB, containing the same concentrations of antibiotics. The inoculate was grown to an OD_{600}=O.4-O.5 and expression of the his-tagged protein was induced with 0.5mM IPTG (Melford) and the culture was incubated for a further 4 to 5 hours. The cells were harvested by centrifugation and resuspended in 50mls Purification Buffer (50mM sodium phosphate, pH 8, 300mM NaCl, 2mM $MgCl_2$, + protease inhibitors). The suspension was snap-frozen and thawed on ice. DNase (5μg/ml), RNase (5μg/ml), and Triton X-100 (TX-100), to a final concentration of 0.1%, was added and incubated rotating at 4^{o}C for 1 hour. The lysate was clarified by centrifugation at 12,000g for 20 minutes, at 4^{o}C. Meanwhile, 5ml of a 50% slurry of Ni^{2+}-agarose (Qiagen) was pre-equilibrated in 10 volumes of ice-cold Purification Buffer, 0.1% TX-100, for 30 minutes. The clarified lysate was incubated with the pre-equilibrated Ni^{2+}-agarose for 1 hour on a rotating wheel at 4^{o}C. The lysate/agarose was centrifuged at 800g, at 4^{o}C, and the Ni^{2+}-agarose was washed 5 times with 5 volumes of Purification Buffer, containing increasing concentrations of imidazole (Sigma) (0mM, 20mM, 40mM, 60mM, and 80mM). The his-tagged protein was then eluted by incubating the agarose pellet for 5 minutes in 1 volume of Purification Buffer containing 250mM imidazole. The elution step was repeated and the eluates were pooled and dialysed extensively against 1X PBS.

Note:- At each stage of the purification an aliquot was taken for analysis by polyacrylamide gel electrophoresis (PAGE).

2.4.1.2 Purification of GST-tagged Proteins

GST-tagged proteins were purified according to the manufacturers protocol (Amersham Pharmacia). Briefly, 400mls of LB/Amp was inoculated with 8ml of an overnight culture of *E.coli,* containing the GST-fusion construct. The protein was

induced with 0.2mM IPTG once the culture reached an OD_{600}=0.5-0.8, and was incubated for a further 4 hours at 37°C. After induction the cells were harvested, resuspended in 20ml of PBS, and snap-frozen. Once thawed, the suspension was sonicated to lyse the bacteria, and incubated with 1% TX-100 for 30 minutes at 4°C. The lysate was clarified by centrifugation at 12,000g, at 4°C. The clarified lysate was incubated with 500µl of pre-equilabrated glutathione-agarose (Sigma), for 1 hour, under rotation at 4°C. The agarose was washed 3 times with 10 volumes of ice-cold PBS, and the fusion protein was eluted, at room temperature for 10 minutes, with 1 volume of elution buffer (10mM reduced-glutathione in 50mM TrisHCl, pH 8.0). The elution step was repeated twice more. The pooled elution fractions were extensively dialysed against 1X PBS.

2.4.2 Generation of Antiserum

To generate RCP and KIAA0941 antiserum, purified his-tagged protein was used to innoculate a New Zealand White rabbit, (Biological Services Unit, U.C.C.). The rabbit was given weekly inoculations of the protein (approx. 250µg, complexed with Freunds' adjuvant, per injection). After four weeks a test bleed was taken and the number of subsequent injections was determined based on the affinity of the serum for low amounts (5-10ng) of the purified protein.

2.4.3 Production of Affinity-Purified Antibodies

Approximately 400µg of protein was separated on a polyacrylamide gel and transferred to nitrocellulose. The protein was revealed with Ponceau S solution and the large band corresponding to the polypeptide was cut out. The Ponceau S was washed off and the band was blocked for 1 hour with 5% fat-free powdered milk (Marvel) dissolved in 1X TBST (10mM Tris pH 8, 150mM NaCl, 0.1% Tween 20). After the blocking step the band was then incubated with 4ml of the rabbit serum, overnight at 4°C. The band was then washed four times with 1X TBST, and the bound antibodies were eluted from the nitrocellulose by incubation with 0.5ml of 0.2M glycine (pH 2.15) for 15 minutes. This was then neutralised with 0.2ml K_2HPO_4. The

elution step was repeated once and the eluates pooled and dialysed extensively in 1X PBS, in the cold-room. The nitrocellulose was conserved at -20^{o}C. The concentration of the affinity-purified antibodies was determined using Bradford reagent.

2.4.4 Subcellular Fractionation

HeLa cells were resuspended in Fractionation Buffer (250mM sucrose, 3mM imidazole, plus protease inhibitors) and lysed by freeze-thawing three times, and passing through a 26-gauge needle six times. A postnuclear supernatant (PNS) was prepared by centrifuging at 10,000g for 10 minutes, at 4^{o}C, in a bench-top refrigerated centrifuge. The PNS was then centrifuged at 100,000g for 1 hour at 4^{o}C in a Beckman L8-55 (rotor Ty65) ultracentrifuge. The cytosol (supernatant) was removed to a fresh tube and the membrane fraction (pellet) was resuspended in an equal volume of Fractionation Buffer. To test the stability of RCP on the membrane, the PNS was incubated with 1% TX-100, 1M Na_2CO_3 (pH11), or 1M NaCl, for 30 minutes on ice prior to ultracentrifugation.

2.4.5 In vitro *Interactions*

2.4.5.1 Radioactive Overlay Method

The protein of interest was separated on an SDS-PAGE gel, and transferred to nitrocellulose. The blot was incubated overnight in Renaturation Buffer (50mM Hepes, pH7.2, 5mM magnesium acetate, 100mM potassium acetate, 3mM DTT, 10mg/ml BSA, 0.1% Triton X-100, 0.3% Tween-20). 35μg of purified His-Rab4AQ67L or GST-Rab11Q70L was loaded with [α-^{32}P] GTP (specific activity 800Ci/mmol), at a final concentration of 0.1μM, in 20mM Hepes pH 7.2, 4mM EDTA and incubated at 37^{o}C for 1 hour. $MgCl_2$ was added to a concentration of 10mM to stabilise the nucleotide on the Rab protein. The nucleotide-loaded protein was then added to 10ml Binding Buffer (12.5mM Hepes, pH7.2, 1.5mM magnesium acetate, 75 mM potassium acetate, 1mM DTT, 2mg/ml BSA, 0.05% TX-100, 0.05% CHAPS) and incubated with the blot, on a rocking platform for 1 hour 15 minutes, at room temperature. The blot was then washed twice for 10 minutes in Wash Buffer (20mM

Tris-HCl, pH7.4, 100mM NaCl, 20mM $MgCl_2$, 0.005% TX-100) and visualised using a phosphor imager (Storm 860, Molecular Dynamics).

2.4.5.2 Far Western Method

The Far Western technique is essentially the same as the radioactive overlay technique, with the exception that GST-tagged Rab GTPases were loaded with cold GTPγS. The incubation and washes of the Rab protein were the same, except the bound protein was visualised with a polyclonal anti-GST antibody (Amersham Pharmacia), using a standard Western blotting procedure.

2.5 Cell Biology

2.5.1 Cell Lines and Antibodies

HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin and streptomycin, and 25mM Hepes, pH 7.4. The BeWo cell line was cultured in HAM'S-F12 supplemented with 10% FCS, 2mM L-glutamine, 25mM Hepes, pH 7.4, and 50U/ml penicillin and streptomycin. The HeLa Rab4WTGFP stable cell line was cultured the same way as untransfected HeLa cells with the exception that the plasmid was maintained with 0.4mg/ml G418, and 20 hours prior to an experiment Rab4WTGFP expression was induced with 10mM sodium butyrate. All cell lines were grown in a 5% CO_2 incubator at 37^{o}C.

The rabbit antibodies used were the affinity-purified H13p antibody, and used at a 1:100 dilution to detect endogenous RCP, and 1:1000 to detect overexpressed protein. The Rab11 antibody was a kind gift from Dr. Bruno Goud and used at a 1:150 dilution. The EEA1 antibody was a gift from Prof. Harald Stenmark, and used at 1:1000. Monoclonal antibodies used included anti-LBPA, a gift from Prof. Jean Greunberg, and used at 1:100. Anti-Express (InVitrogen) was used at 1:2000. The transferrin receptor antibody (Roche) for immunofluorescence was used at 1:1000. The anti-TfnR antibody for immunoblotting was from Zymed and used at 1:1000.

Anti-β tubulin (Sigma) used at 1:300. Anti-EEA1 (BD Transduction Laboratories) used at 1:1000.

2.5.2 Transfection

Transfection of HeLa cells, for the study of the effects of overexpression of H13p, RCP, or KIAA0941, used the T7 RNA polymerase recombinant vaccinia virus system. This method resulted in a high level of expression of the recombinant protein. Cells were seeded on coverslips in 24-well plates so that they reached a confluency of 60-70% the next day. They were then infected at a multiplicity of infection (MOI) of 10 with vaccinia strain MVA T7 polymerase. After 45 minutes the cells were washed once with 1X PBS and then transfected with 1.5-3μg of plasmid DNA, in serum free medium. The plasmid transfection mix was prepared as follows; the plasmid DNA was diluted to 20μl in HBS (20mM Hepes, pH 7.2, 150mM NaCl). 10μl of DOTAP (Roche) was mixed with an equal volume of HBS and added to the DNA solution. The solution was mixed by pipetting several times. A DOTAP/DNA complex was allowed to form by incubating at room temperature for 15 minutes. The DNA/DOTAP mixture was then diluted in 200μl of DMEM without serum, supplemented with 0.2% BSA, and added to the infected cells. The transfections were allowed to proceed for 6-7 hours.

In some cases, for colocalisation purposes, Effectene (Qiagen), a non-liposomal transfection reagent, was used. The manufacturer's protocol was followed. Transfections usually proceeded for 6-8 hours, which gave an expression level slightly above that of the endogenous protein.

2.5.3 Immunofluorescence

Cells on 11mm round glass coverslips were fixed with 3% paraformaldehyde. Free aldehyde groups were quenched with 50mM NH_4Cl. The cells were permeablised with 0.05% saponin in 1X PBS, 0.2% BSA, and then incubated in a humid chamber with the primary antibodies, for 1 hour. The coverslips were then washed with 1X PBS and incubated with secondary antibodies. The secondary

antibodies used were Texas Red-coupled donkey anti-rabbit IgG or anti-mouse IgG, fluorescein isothiocyanate (FITC)-coupled donkey anti-mouse IgG, and AMCA-coupled donkey anti-rabbit IgG (Jackson Immunochemicals). All secondary antibodies were used at a 1 in 200 dilution. Coverslips were mounted on Mowiol and examined on an Eclipse E600 (Nikon) immunofluorescence microscope, or a confocal microscope (Zeiss). Note: When the β-tubulin antibody was used the cells were fixed with -20^{o}C methanol.

2.5.4 Preparation of FITC-Transferrin

FITC-conjugated transferrin was produced by preparing 100µl of a 5mg/ml solution of holo-transferrin (Sigma), in PBS plus 1mM $MgCl_2$, 1mM $CaCl_2$ (PBS^{++}). 50µl of 1M $NaHCO_3$, pH 8.5, and 150µl of a 10mg/ml stock of FITC-Celite (Calbiochem) in PBS^{++}, was added and brought to a final volume of 225µl with PBS^{++}. This was incubated for 30 minutes at room temperature. To stop the reaction 6.5µl of 1M glycine was added. The reaction was centrifuged to remove the Celite, and the supernatant was loaded onto a PD-10 column (Pharmacia) that had been equilibrated with 50mM Hepes, pH7.25, 100mM NaCl. The supernatant separated into two yellow bands and the lower band was collected.
Note: At all stages the FITC-transferrin was protected from light in amber 1.5ml microcentrifuge tubes.

2.5.5 Uptake of FITC-Tfn and Texas-Red EGF in HeLa Cells

HeLa cells were seeded on glass coverslips so that they were approximately 70% confluent the next day. The cells were washed once with PBS and then starved in DMEM lacking serum for at least two hours at 37^{o}C (in order to deplete the cells of endogenous transferrin). In cells that had been transfected, prior to ligand uptake, this step was omitted, as the transfection medium contained no serum. The coverslips were transferred to a humid chamber and incubated at 37^{o}C (5% CO_2) with 25µg/ml of FITC-transferrin in Depletion Media (DMEM, 25mM Hepes pH7.2, 1% BSA) for one hour, to load the recycling pathway. In the cases where there was dual ligand

uptake, Texas Red-labelled EGF (Molecular Probes) was used at a concentration of 3μg/ml. After the incubation, the cells were washed twice with ice-cold 1X PBS and fixed with ice-cold 3% paraformaldehyde. They were then processed for immunofluorescence microscopy as described previously.

For the pulse/chase experiments the transfections were performed in duplicate. After transfection, the ligands were internalised at 18^{o}C for 45 minutes. One duplicate was washed and immediately fixed, while for the other duplicate, the ligands were chased into the cell at 37^{o}C for 10 minutes, prior to fixation.

2.5.6 In Vitro *Recycling Assay*

The *in vitro* recycling assays have been described elsewhere (Ullrich *et al.*, 1996). In brief, BHK cells infected with the modified vaccinia virus were co-transfected with the human TfnR and the indicated construct. Control cells were transfected with the human TfnR alone. ^{125}I-Tfn was allowed to bind to the cell surface, on ice for 1 hour. Excess ligand was washed off and the ^{125}I-Tfn was internalised for the indicated times. The amount of ^{125}I-Tfn in the culture medium, and therefore recycled, was calculated, and expressed as a percentage of total ^{125}I-Tfn (cell associated and culture medium ^{125}I-Tfn).

To measure the kinetics of recycling specifically from the recycling compartment, the above protocol was modified. The transfected BHK cells were pulsed at 37^{o}C for minutes with ^{125}I-Tfn, excess ligand was washed away, and the cells were then chase at 37^{o}C for a further 30 minutes. This allowed the recycling compartment to be loaded. The amount of ^{125}I-Tfn in the culture medium at the indicated time points, after the chase was calculated and expressed as a percentage of the total ^{125}I-Tfn.

For both assays, the effects on the rate of recycling due to transfection of the indicated mutants was compared to the kinetics of the control assay.

3 Results

3.1 Rab Coupling Protein

3.1.1 Yeast Two-Hybrid Screen of an oligo-dT Primed HeLa cDNA Library using Rab4Q67L as Bait

Rab4 is a small GTPase implicated in the regulation of transport from the early sorting endosome. In contrast to Rab GTPases such as Rab5 and Rab6, relatively little is known about Rab4. In order to identify effector proteins for Rab4, a yeast two-hybrid screen was performed using a constitutively active mutant of Rab4, Rab4Q67L (-CXC), as bait (for a complete description refer to the PhD thesis of Alan Hendrick). Rab4Q67L has a point mutation in its GTPase domain that results in the reduced ability of the mutant to hydrolyse GTP to GDP. An oligo-dT primed HeLa cDNA library, which had been used successfully in other yeast two-hybrid assays (Stenmark *et al.,* 1995; Cantalupo *et al.,* 2001), was screened with the bait. *Saccharomyces cerevisiae* L40 was transformed with the Rab4Q67L mutant in a vector that expresses it as a fusion with the DNA binding domain of the LexA transcription factor, pVJL10 Rab4Q67L(-CXC). This mutant lacked the CXC prenylation motif, since communication with other labs (M.Zerial and H.Stenmark) suggested that the presence of the geranyl-geranyl group results in a high background of false-positive interactions. Transformants were selected on media lacking tryptophan. Yeast containing the bait vector was then transformed with the HeLa cDNA library and plated on media lacking tryptophan, leucine, and histidine. Only yeast colonies that contain a library clone that interacts with the Rab4Q67L(-CXC) fusion protein, allowing the DNA binding domain of the bait and the activation domain fused to the prey to come into close proximity and activate transcription of the HIS3 gene, can grow on the selective media.

Approximately $3x10^6$ transformants were screened, of which 44 colonies grew on the His^- media and activated the β-galactosidase reporter gene. Plasmids were recovered from these colonies using a plasmid preparation protocol modified for yeast, and the library plasmid was selected in the *E.coli* HB101 strain. To further analyse these library clones and identify false-positives they were co-transformed into L40 along with Rab4Q67L or lamin. Lamin is a nuclear structural protein that was used as a negative control. Lamin is unrelated to Rab4 and therefore any of the library

clones that interacted with it were considered to be false-positives. Fourteen library clones expressed the lacZ gene specifically with Rab4Q67L. These positive clones were sequenced. Five clones encoded Rabaptin-5, a Rab5 effector protein that had previously been shown to interact with Rab4 (Vitale *et al.,* 1998). Four clones encoded a novel protein that showed no homology with any protein in the databases, at the time. The remaining plasmids were only represented once and contained unrelated reading frames.

Clone H13, a member of the second group of clones, was chosen for further study. All the clones in this group contained an approximately 3.6kb insert. Digestion of clone H13 with *EcoRI* cut out three insert fragments of just under 1kb, 550bp, and approximately 2.1kb, plus the vector backbone that runs at over 7kb;

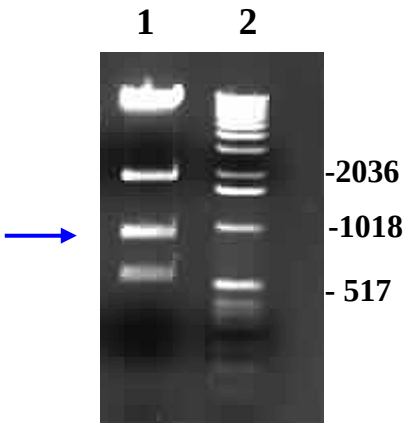

Figure 8. *EcoRI* digestion of clone H13. Clone H13 digested with *EcoRI*. The arrow indicates the fragment that contains the H13 coding sequence (lane 1). DNA molecular weight markers (lane 2).

Sequencing revealed that the 1kb EcoRI fragment contained a 270 amino acid reading frame that was expressed 'in frame' with the Gal4 activation domain of pGADGH (Figure 9). The 550bp fragment contained 3'-untranslated sequence followed by a polyA tail. Surprisingly, the 2kb fragment contained a yeast transposable element that is likely to have transposed into the cDNA during the construction, or screening of the library. To ensure that the interaction with Rab4Q67L was not due to the presence of the transposon, the 1kb *EcoRI* fragment, alone, was subcloned into the prey vector, and tested for interaction with Rab4. As expected, there was still an interaction with Rab4 (data not shown).

```
  1 - CTTCCACCAAGGACTCCTTGAAGTCTATGACCTTGCCGACCTACCGACCTGCCCCACTGA - 60
    -   S  T  K  D  S  L  K  S  M  T  L  P  T  Y  R  P  A  P  L  I
 61 - TCAGTGGGGACCTCAGGGAAAAAATGGCCCCCGCAAACTCAGAGGCCACAAAAGAAGCTA - 120
    -   S  G  D  L  R  E  K  M  A  P  A  N  S  E  A  T  K  E  A  K
121 - AGGAGAGCAAGAAGCCAGAGAGCAGGAGGTCCTCTTTGCTGTCTCTGATGACGGGGAAGA - 180
    -   E  S  K  K  P  E  S  R  R  S  S  L  L  S  L  M  T  G  K  K
181 - AGGATGTGGCTAAGGGCAGTGAAGGTGAAAACCTTCTCACGGTCCCAGGGAGGGAGAAGG - 240
    -   D  V  A  K  G  S  E  G  E  N  L  L  T  V  P  G  R  E  K  E
241 - AAGGCATGCTGATGGGGGTTAAGCCGGGGGAGGACGCATCGGGGCCTGCTGAAGACCTTG - 300
    -   G  M  L  M  G  V  K  P  G  E  D  A  S  G  P  A  E  D  L  V
301 - TGAGAAGATCTGAGAAAGATACTGCAGCTGTTGTCTCCAGACAGGGCAGCTCCCTGAACC - 360
    -   R  R  S  E  K  D  T  A  A  V  V  S  R  Q  G  S  S  L  N  L
361 - TCTTTGAAGATGTGCAGATCACAGAACCAGAAGCTGAGCCAGAGTCCAAGTCTGAACCGA - 420
    -   F  E  D  V  Q  I  T  E  P  E  A  E  P  E  S  K  S  E  P  R
421 - GACCTCCAATTTCCTCTCCGAGGGCTCCCCAGACCAGAGCTGTCAAGCCCCGACTTCATC - 480
    -   P  P  I  S  S  P  R  A  P  Q  T  R  A  V  K  P  R  L  H  P
481 - CTGTGAAGCCAATGAATGCCACGGCCACCAAGGTTGCTAACTGCAGCTTGGGAACTGCCA - 540
    -   V  K  P  M  N  A  T  A  T  K  V  A  N  C  S  L  G  T  A  T
541 - CCATCATCAGTGAGAACTTGAACAATGAGGTCATGATGAAGAAATACAGCCCCTCGGACC - 600
    -   I  I  S  E  N  L  N  N  E  V  M  M  K  K  Y  S  P  S  D  P
601 - CTGCATTTGCATATGCGCAGCTGACCCACGATGAGCTGATTCAGCTGGTCCTCAAACAGA - 660
    -   A  F  A  Y  A  Q  L  T  H  D  E  L  I  Q  L  V  L  K  Q  K
661 - AGGAAACGATAAGCAAGAAGGAGTTCCAGGTCCGCGAGCTGGAAGACTACATTGACAACC - 720
    -   E  T  I  S  K  K  E  F  Q  V  R  E  L  E  D  Y  I  D  N  L
721 - TGCTTGTCAGGGTCATGGAAGAAACCCCCAATATCCTCCGCATCCCGACTCAGGTTGGCA - 780
    -   L  V  R  V  M  E  E  T  P  N  I  L  R  I  P  T  Q  V  G  K
781 - AAAAAGCAGGAAAGATGTAAATCAGCAGAAAAAAAACACCGAGACGTTTCTGTGACTTCA - 840
    -   K  A  G  K  M  *
841 - CTTTCACCTGCTCCAGGGGTCAAGGACTTGCCTTGCCTGATAACCAGCCAGCAGGCTCCG - 900
901 - AATCACCATCTCCCTCACATGTTATCCGGCAAGAGTGAATTC - 942
```

Figure 9. DNA sequence and translation of the clone H13 *EcoRI* fragment that contains the reading frame. The downstream *EcoRI* site is in bold.

3.1.2 Clone H13 Interacts in a Nucleotide-Dependent Manner with Rab4 and Rab11

The Rab4 interaction was tested to determine if clone H13 had a preference for the nucleotide-bound state of the small GTPase. *S. cerevisiae* L40 was co-transformed with clone H13 and either Rab4Q67L, Rab4WT, Rab4S22N (GDP-locked mutant), or a constitutively active form of Rab4 with a mutation in the putative effector domain, Rab4Q67L/I41E. Colonies from each transformation plate were spotted in parallel onto media containing histidine, and media lacking histidine. There was strong growth with Rab4Q67L, slightly less with Rab4WT, and no growth with Rab4S22N, or the effector mutant (Figure 10A & 10C). Thus, clone H13 interacts preferentially with the active form of Rab4, and this interaction is mediated via the putative 'effector' domain. To determine if clone H13 interacts exclusively with Rab4, it was tested for interactions with the constitutively active mutants of Rab3, Rab5, Rab6 and Rab7. There was no interaction with any of these small GTPases (Figure 10C), however,

there was a strong interaction with Rab11 (Figure 10B & 10C). The interaction between clone H13 and Rab11 did not appear to be nucleotide-dependent and was not affected by a mutation in the Rab11 effector domain;

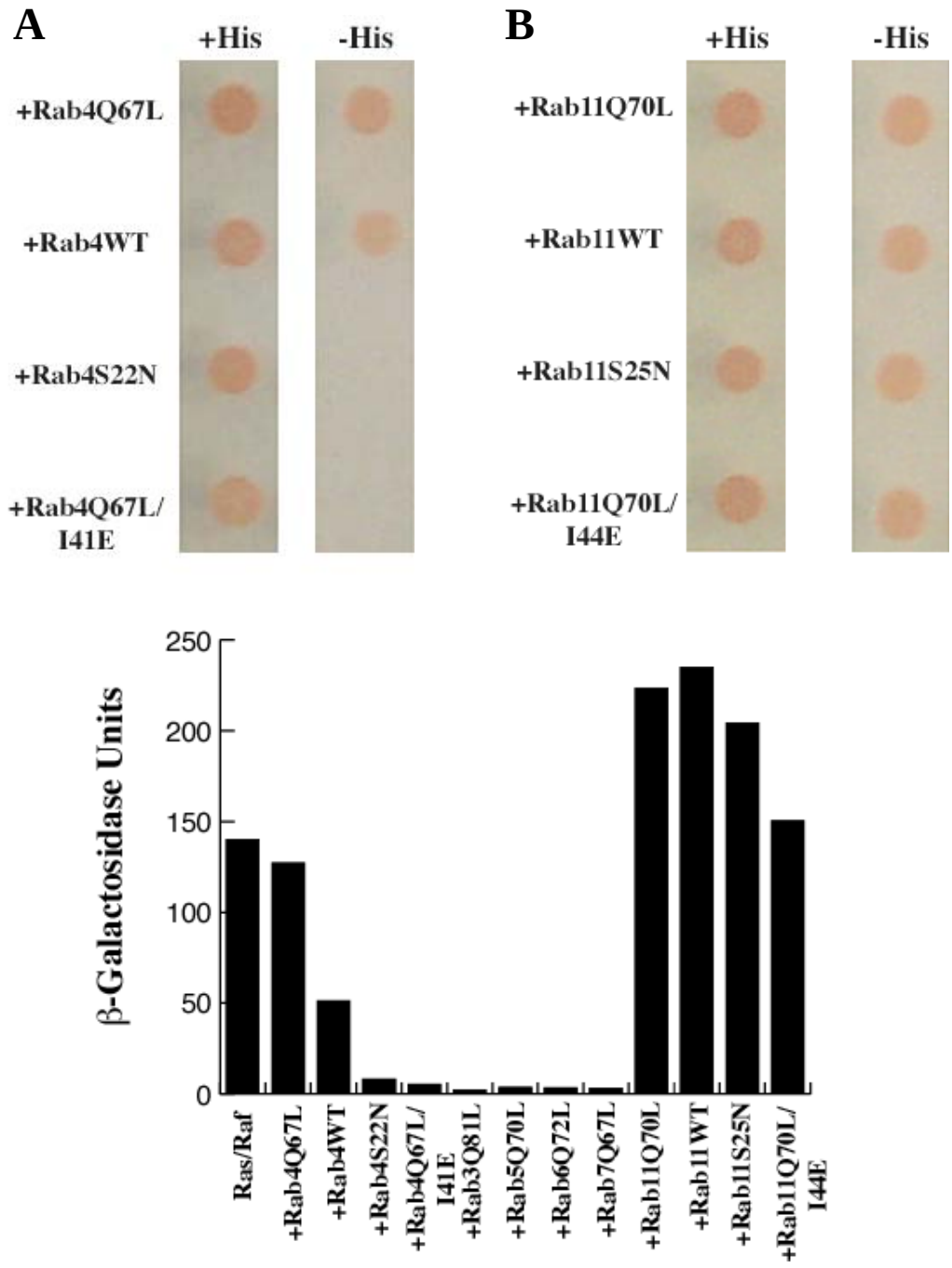

Figure 10. Interaction of clone H13 with Rab4, and its mutants **(A)**, or with Rab11, and its mutants **(B)**. Growth on media lacking histidine indicates an interaction between the 'bait' and 'prey' fusion proteins. Quantitative β-galactosidase assays. Clone H13 was co-transformed with the indicated Rab constructs and the β-galactosidase activity of the double transformants was measured using ONPG as substrate **(C)**. The Ras/Raf interaction was used as a positive control.

The strength of the interactions between clone H13, and Rab4 and its mutants, or Rab11 and its mutants, was quantified in a liquid β-galactosidase assay (Figure 10C). An interaction between 'bait' and 'prey' vectors results in the transcription of the HIS3 and lacZ reporter genes. The strength of an interaction can be quantified by measuring the β-galactosidase activity in a lysate, containing two interacting proteins, using *o*-nitrophenyl-β-D-galactosidase (ONPG) as substrate. Clone H13 displayed a stronger, overall, interaction with all the Rab11 constructs, than it did with Rab4Q67L.

Taken together these results indicate that clone H13 is likely to be a genuine Rab4 interacting polypeptide, and its preference for the GTP-bound form of Rab4 fits the conventional model of a Rab effector. The interaction with Rab11 is also likely to be of importance, despite the somewhat different interaction pattern, as clone H13 does not interact with other closely related Rab GTPases, ruling out the possibility that it is just a 'sticky' protein. Even though the interaction with Rab11 was unexpected, it is not unprecedented. Rabaptin-5 interacts with both Rab5 and Rab4 (Vitale *et al.,* 1998). Since Rab4 functions upstream of Rab11 along the receptor recycling pathway, it is possible that clone H13 represents a protein that acts as an intermediate between a Rab4-regulated transport event and a Rab11-regulated transport event.

3.1.2.1 Generation of a Rab11 Effector Domain Mutant

The yeast two-hybrid Rab11 wild-type, and mutant, constructs were a kind gift from Ms. Francesca Senic-Matuglia and Dr Bruno Goud. However, since a Rab11 effector domain mutant was not available, it was necessary to generate this mutant, by two step site-directed mutagenesis. The effector domains of Rab proteins are located within their 'switch' regions (Stenmark *et al.,* 2001). The corresponding mutation to the Rab4I41E mutant was introduced into Rab11;

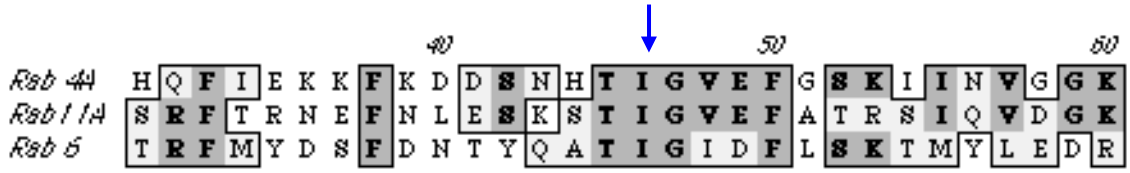

Figure 11. ClustalW alignment of the effector domains of Rab4, Rab11, and Rab6. The arrow indicates the position of the isoleucine to be mutated.

The isoleucine at position 44 was to be mutated to a glutamic acid residue. The constitutively active mutant, Rab11Q70L, was used as template for the reaction. The first round PCR contained the mutagenic primer and an upstream vector based primer. The 150bp product of this reaction was used as the upstream primer in the second round PCR;

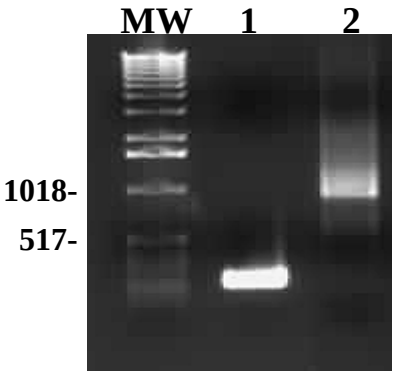

Figure 12. Generation of the Rab11Q70L/I44E mutant. (1) 1st-round PCR product; (2) 2nd-round PCR product.

The second-round product was digested and ligated into the yeast two-hybrid bait vector, pLexA. The introduction of the point mutation, and the absence of other mutations introduced during the PCR process, was confirmed by sequencing (Dept. Biochemistry, University of Oxford).

3.1.3 Expression and Purification of Clone H13 Protein

To perform biochemical studies, and to raise antibodies, it was necessary to express and purify H13 polypeptide (H13p). To this end, the 1kb fragment containing the H13 reading frame was subcloned into a prokaryotic expression vector, pTrcHisC (Invitrogen). This vector adds an amino-terminal epitope containing six consecutive histidine residues to the protein of interest. The 6XHis-tag has a high affinity for nickel. This allows for the purification of the fusion protein from a bacterial cell lysate using Ni^{2+}-chelated agarose. The 6XHis-tag has a low antigenicity making it an ideal tag for the production of protein that will be used for generating antiserum. Expression of 6XHis-H13p in *E.coli* BL21 was induced with 0.2mM IPTG. Five hours post-induction a bacterial cell lysate was prepared and incubated with Ni^{2+}-agarose. After several washes the bound protein was eluted with a high concentration

of imidazole. This system allowed for the purification of sufficiently pure H13 fusion polypeptide to inoculate a rabbit and use for biochemical studies. The H13 fusion construct expressed a 37kDa protein that was used to inoculate a New Zealand White rabbit, and the resulting affinity purified antibodies could detect as little as 5ng of purified H13p by Western blot;

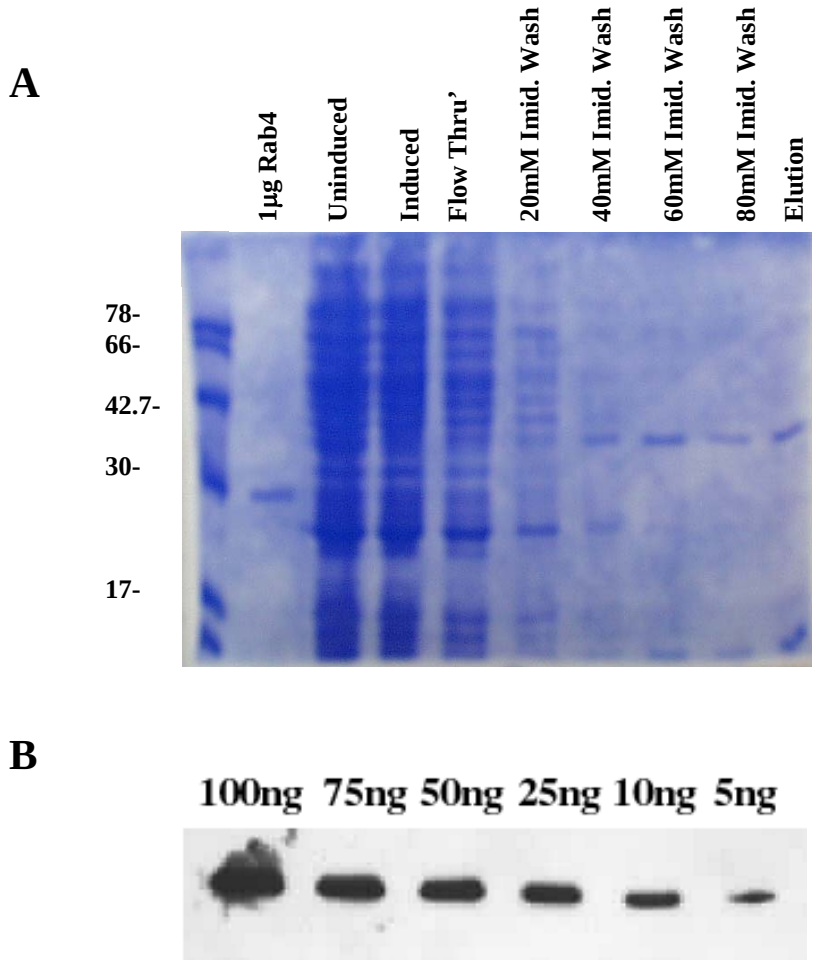

Figure 13. A. Coomassie blue gel of the steps involved in the purification of 6XHis-H13p, **B**. Western blot showing the detection limits of the H13p affinity-purified antibody.

The 6XHis-tag allowed for the relatively rapid and simple purification of large quantities of pure H13p. The resulting H13p antiserum was useful for detecting overexpressed H13p, however, to detect the endogenous protein by immunoblot or immunofluorescence it was necessary to affinity purify the serum. Analysis revealed that it was specific for H13p since it did not recognise the 6XHis tag of other proteins (data not shown).

3.1.4 H13p Antiserum Detects a Ubiquitously Expressed 80kDa Protein

To determine the size and expression pattern of endogenous H13p, affinity purified H13p antibody was used to probe the post-nuclear supernatants (PNS) of various tissue culture cell lines. The antibody detected a single band that migrated at approximately 80kDa, in all the cell lines tested;

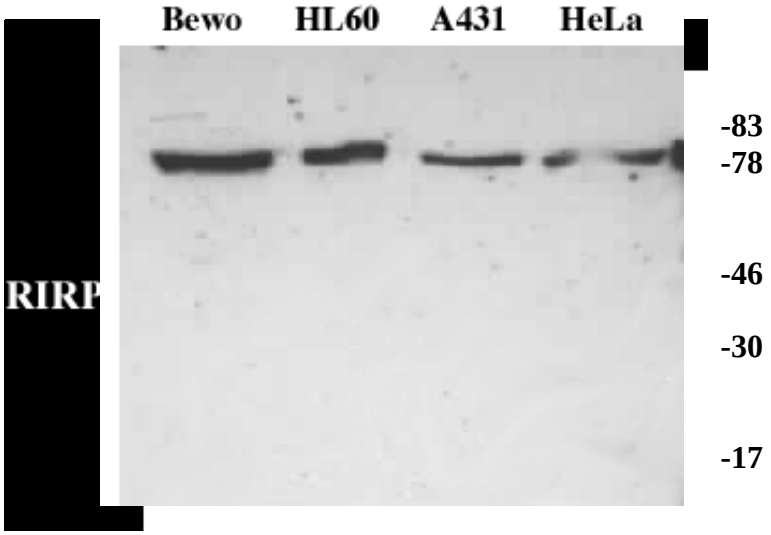

Figure 14. Affinity purified H13p antibody detects an 80kDa protein expressed in BeWo (placental trophoblastic choriocarcinoma) cells, HL60 (promyelocytic monocyte) cells, A431 (epidermoid carcinoma) cells, and HeLa (cervical carcinoma) cells

This result indicates that clone H13 is only a partial clone. The method of construction of the two-hybrid library, i.e. oligo-dT primed, and the presence of the polyA-tail in the H13 cDNA, indicates that H13 represents the carboxy-terminal portion of a full-length protein. The full-length 80kDa protein was named Rab Coupling Protein (RCP), since it interacts with two Rab GTPases, which play sequential roles in the receptor recycling pathway. (See the next section for a description of the cloning of the full-length cDNA). RCP appears to be expressed in most tissues indicating that it plays an important general role along the receptor recycling pathway rather than a tissue-specific role.

3.1.5 Generation of the Full-Length RCP cDNA

Since the H13 fusion construct codes for an approximately 37kDa protein, it was apparent that it represents only a portion of the full-length RCP gene. In order to clone the full-length RCP cDNA a number of avenues were followed. These included searching the expressed sequence tag (EST) databases, screening a λ cDNA library, using the rapid amplification of cDNA ends (RACE) PCR-based method, and finally a bioinformatic approach, that eventually identified the full-length cDNA. Initially, searching the EST databases with the H13 cDNA sequence revealed several homologous EST clones. One of these, IMAGE Clone 150706, contained an insert cDNA that encompassed the entire H13 sequence and approximately 1kb of upstream sequence, which included a potential ATG start codon at the 5'-end. There were no other ESTs in the databases that provided any further 5' sequence. The reading-frame of IMAGE 150706 was amplified using a proof-reading DNA polymerase and cloned into the pGADGH two hybrid 'prey' vector. Two-hybrid analysis showed that it interacted with both Rab4 and Rab11. The cDNA was then cloned into the pcDNA3.1HisB vector and expressed in HeLa cells. The exogenous protein displayed a similar distribution to endogenous RCP, and was detectable by the H13p affinity purified antibody (data not shown). However, when a postnuclear supernatant (PNS) was prepared from HeLa cells expressing the EST cDNA, there was a discrepancy in the size of the recombinant protein compared with that of endogenous RCP. The recombinant protein migrated slightly lower than endogenous RCP suggesting that the EST was missing the extreme 5'-end of RCP. The cDNA cloned from this EST was thus designated δRCP, reflecting the fact that it was an incomplete cDNA.

To clone the full-length RCP cDNA it was initially decided to use the RACE technique as this approach could potentially deliver the RCP 5'-end in a short time. Since we estimated that we were missing no more than 300bp of coding sequence, the PCR reactions would not have the added complication of requiring large products to be amplified. An adaptor-ligated placental cDNA library was generated from BeWo mRNA (courtesy of Alan Hendrick), using the Marathon-Ready cDNA kit (Clontech). This library consisted of cDNAs with short adaptor sequences ligated to their 5'- and 3'- ends. A primer that binds to the adaptor, used in conjunction with a primer that is specific for the gene of interest, should allow the amplification of a 5'- product or a

3'- product, depending on the direction of the gene-specific primer (see schematic in Materials & Methods). Gene-specific primers were designed close to the 5'-end of the known, δRCP, sequence and were used in 5'RACE reactions. However, despite numerous conditions, annealing temperatures, cycle numbers, nested PCRs, Mg^{2+} concentrations, no product was amplified that provided sequence upstream of the sequence that was already cloned in δRCP. Several times 5'RACE products were amplified and sequenced that contained no more than 10bp of upstream sequence. Since the RACE technique was unsuccessful a 5'-STRETCH λ HeLa cDNA library (Clontech) was purchased and a conventional phage library screen was performed using radiolabeled H13 cDNA as a probe. This library was constructed in such a way that there was a bias towards full-length inserts. Two positive plaques were identified, from which the phage were rescued and their inserts analysed. However, while they contained the entire H13 cDNA they did not provide the 5'-end of RCP.

Meanwhile, two major pieces of work were made public that finally allowed the identification of the full-length RCP gene. The complete human genome sequence was released (Lander *et al.,* 2001; Venter *et al.,* 2001), and the RIKEN Mouse Gene Encyclopaedia Project reported the characterisation of a set of 21,076 full-length mouse cDNAs (Kawai *et al.,* 2001). The δRCP sequence was used in a 'BLAST' search of the consensus human genome sequence and the results revealed that the RCP gene was present on the short arm of chromosome 8, 8p11.2, and was divided into at least four exons. However, it was not possible to identify the missing upstream coding sequence. Searching the RIKEN database with the δRCP sequence identified a mouse transcript, AK014696, which was highly homologous to δRCP. The mouse transcript contained an ORF that coded for a 645 amino acid protein that displayed 80% homology (and 8% similarity) with δRCPp (Figure 17). It was concluded that AK014696 was the mouse homologue of RCP, and was designated mRCP. mRCP contained 269bp of coding sequence, and a Kozak's consensus sequence, upstream from the beginning of the δRCP sequence. This 269bp mRCP sequence, when used to search the human chromosome 8 sequence, identified the first exon of human RCP. Thus, from this bioinformatic approach it was possible to determine the full-length RCP coding sequence. Fortunately, an EST recently deposited in the IMAGE databank, IMAGE Clone 3956619, contained the previously unidentified 5'-sequence. This EST was requested and sequenced. It contained a large 4.5kb insert and coded

for the entire RCP open reading frame (Figure 15). The reading frame was subcloned into the pGEM1 vector and expressed in HeLa cells using the vaccinia system. Comparison of the molecular weights of the endogenous RCP and recombinant RCP revealed that they migrated at the same molecular weight (Figure 16). RCP is comprised of 5 exons at locus 8p11.2. One of the difficulties in identifying the 5'-end sequence was that it is coded by a single exon located over 100kb upstream from the second exon.

As a footnote to this section, analysis of the RCP sequence identified a NotI restriction site just upstream of the start of the δRCP sequence. This could explain the difficulty in cloning the full-length RCP cDNA from the RACE library and the λ HeLa cDNA library. NotI is commonly used in the construction of libraries, and in this case its use would inadvertently cut off the 5'end of the cDNA.

```
  1 - CCCGCTTCTGGAGTGTTATCGTCACCATGTCCCTAATGGTCTCGGCTGGCCGGGGCCTGG - 60
    -                           M  S  L  M  V  S  A  G  R  G  L  G
 61 - GGGCCGTGTGGTCCCCAACCCACGTGCAGGTGACGGTGCTGCAGGCGCGGGGCCTGCGGG - 120
    -   A  V  W  S  P  T  H  V  Q  V  T  V  L  Q  A  R  G  L  R  A
121 - CCAAGGGCCCCGGGGGCACGAGCGACGCGTACGCGGTGATCCAGGTGGGCAAGGAGAAGT - 180
    -   K  G  P  G  G  T  S  D  A  Y  A  V  I  Q  V  G  K  E  K  Y
181 - ACGCCACCTCCGTGTCGGAGCGCAGCCTGGGCGCGCCCGTGTGGCGCGAGGAGGCCACCT - 240
    -   A  T  S  V  S  E  R  S  L  G  A  P  V  W  R  E  E  A  T  F
241 - TCGAGCTGCCATCGCTGCTGTCCTCCGGACCCGCGGCCGCCGCCACCCTGCAGCTCACCG - 300
    -   E  L  P  S  L  L  S  S  G  P  A  A  A  A  T  L  Q  L  T  V
301 - TGCTGCACCGCGCGCTGCTCGGCCTCGACAAGTTCCTGGGCCGCGCCGAGGTGGACCTGC - 360
    -   L  H  R  A  L  L  G  L  D  K  F  L  G  R  A  E  V  D  L  R
361 - GGGATCTGCACCGCGACCAGGGCCGCAGGAAGACGCAGTGGTATAAGTTGAAATCCAAAC - 420
    -   D  L  H  R  D  Q  G  R  R  K  T  Q  W  Y  K  L  K  S  K  P
421 - CAGGAAAGAAGGACAAGGAGCGAGGAGAAATTGAGGTTGACATCCAGTTTATGAGAAACA - 480
    -   G  K  K  D  K  E  R  G  E  I  E  V  D  I  Q  F  M  R  N  N
481 - ACATGACTGCCAGCATGTTTGACCTTTCTATGAAAGACAAGTCTCGGAATCCATTTGGAA - 540
    -   M  T  A  S  M  F  D  L  S  M  K  D  K  S  R  N  P  F  G  K
541 - AGCTGAAGGACAAGATCAAGGGGAAGAATAAGGACAGTGGGTCAGACACCGCCTCCGCCA - 600
    -   L  K  D  K  I  K  G  K  N  K  D  S  G  S  D  T  A  S  A  I
601 - TCATCCCTAGCACGACACCTTCGGTCGACAGTGATGATGAGTCTGTGGTTAAAGACAAGA - 660
    -   I  P  S  T  T  P  S  V  D  S  D  D  E  S  V  V  K  D  K  K
661 - AAAAGAAATCAAAGATCAAGACCTTACTTTCCAAGTCAAATTTGCAGAAGACGCCTCTTT - 720
    -   K  K  S  K  I  K  T  L  L  S  K  S  N  L  Q  K  T  P  L  S
721 - CCCAGTCCATGTCTGTCCTGCCGACTTCAAAGCCAGAAAAAGTGCTGCTTCGTCCCGGAG - 780
    -   Q  S  M  S  V  L  P  T  S  K  P  E  K  V  L  L  R  P  G  D
781 - ACTTTCAGTCCCAGTGGGATGAAGATGACAATGAGGATGAGTCCTCCTCGGCCTCGGATG - 840
    -   F  Q  S  Q  W  D  E  D  D  N  E  D  E  S  S  S  A  S  D  V
841 - TCATGTCTCACAAGAGAACAGCGAGTACGGATCTTAAGCAACTGAACCAGGTCAACTTTA - 900
    -   M  S  H  K  R  T  A  S  T  D  L  K  Q  L  N  Q  V  N  F  T
901 - CCCTTCCCAAGAAGGAAGGACTTTCCTTTCTTGGTGGCCTTCGGTCTAAGAATGATGTCC - 960
    -   L  P  K  K  E  G  L  S  F  L  G  G  L  R  S  K  N  D  V  L
961 - TTTCCCGCTCTAATGTCTGCATCAATGGGAACCATGTTTACCTGGAGCAGCCCCAACCCA - 1020
    -   S  R  S  N  V  C  I  N  G  N  H  V  Y  L  E  Q  P  Q  P  T
1021 - CCGGTGAGATCAAGGATAGCAGCCCGTCCTCCTCCCCATCCCCCAAGGGTTTCAGAAAGA - 1080
    -   G  E  I  K  D  S  S  P  S  S  S  P  S  P  K  G  F  R  K  K
1081 - AAACATTGTTCTCTTCTACAGAGAACCTGGCGGCTGGGTCTTGGAAGGAGCCTGCTGAAG - 1140
    -   T  L  F  S  S  T  E  N  L  A  A  G  S  W  K  E  P  A  E  G
1141 - GAGGTGGGCTGTCTACTGACAGGGATGTCTCCGAATCTTCCACCAAGGACTCCTTGAAGT - 1200
    -   G  G  L  S  T  D  R  D  V  S  E  S  S  T  K  D  S  L  K  S
1201 - CTATGACCTTGCCGACCTACCGACCTGCCCCACTGATCAGTGGGGACCTCAGGGAAAAAA - 1260
    -   M  T  L  P  T  Y  R  P  A  P  L  I  S  G  D  L  R  E  K  M
1261 - TGGCCCCCGCAAACTCAGAGGCCACAAAAGAAGCTAAGGAGAGCAAGAAGCCAGAGAGCA - 1320
    -   A  P  A  N  S  E  A  T  K  E  A  K  E  S  K  K  P  E  S  R
1321 - GGAGGTCCTCTTTGCTGTCTCTGATGACGGGGAAGAAGGATGTGGCTAAGGGCAGTGAAG - 1380
    -   R  S  S  L  L  S  L  M  T  G  K  K  D  V  A  K  G  S  E  G
1381 - GTGAAAACCTTCTCACGGTCCCAGGGAGGGAGAAGGAAGGCATGCTGATGGGGGTTAAGC - 1440
    -   E  N  L  L  T  V  P  G  R  E  K  E  G  M  L  M  G  V  K  P
1441 - CGGGGGAGGACGCATCGGGGCCTGCTGAAGACCTTGTGAGAAGATCTGAGAAAGATACTG - 1500
    -   G  E  D  A  S  G  P  A  E  D  L  V  R  R  S  E  K  D  T  A
1501 - CAGCTGTTGTCTCCAGACAGGGCAGCTCCCTGAACCTCTTTGAAGATGTGCAGATCACAG - 1560
    -   A  V  V  S  R  Q  G  S  S  L  N  L  F  E  D  V  Q  I  T  E
1561 - AACCAGAAGCTGAGCCAGAGTCCAAGTCTGAACCGAGACCTCCAATTTCCTCTCCGAGGG - 1620
    -   P  E  A  E  P  E  S  K  S  E  P  R  P  P  I  S  S  P  R  A
1621 - CTCCCCAGACCAGAGCTGTCAAGCCCCGACTTCATCCTGTGAAGCCAATGAATGCCACGG - 1680
    -   P  Q  T  R  A  V  K  P  R  L  H  P  V  K  P  M  N  A  T  A
1681 - CCACCAAGGTTGCTAACTGCAGCTTGGGAACTGCCACCATCATCAGTGAGAACTTGAACA - 1740
    -   T  K  V  A  N  C  S  L  G  T  A  T  I  I  S  E  N  L  N  N
1741 - ATGAGGTCATGATGAAGAAATACAGCCCCTCGGACCCTGCATTTGCATATGCGCAGCTGA - 1800
    -   E  V  M  M  K  K  Y  S  P  S  D  P  A  F  A  Y  A  Q  L  T
1801 - CCCACGATGAGCTGATTCAGCTGGTCCTCAAACAGAAGGAAACGATAAGCAAGAAGGAGT - 1860
    -   H  D  E  L  I  Q  L  V  L  K  Q  K  E  T  I  S  K  K  E  F
1861 - TCCAGGTCCGCGAGCTGGAAGACTACATTGACAACCTGCTTGTCAGGGTCATGGAAGAAA - 1920
    -   Q  V  R  E  L  E  D  Y  I  D  N  L  L  V  R  V  M  E  E  T
1921 - CCCCCAATATCCTCCGCATCCCGACTCAGGTTGGCAAAAAAGCAGGAAAGATGTAAATCA - 1980
    -   P  N  I  L  R  I  P  T  Q  V  G  K  K  A  G  K  M  *
1981 - GCAGAAAAAAAACACCGAGACGTTTCTGTGACTTCACTTTCACCTGCTCCAGGGGTCAAG - 2040
2041 - GACTTGCCTTGCCTGATAACCAGCCAGCAGGCTCCGAATCACCATCTCCCTCACATGTTA - 2100
2101 - TCCGGCAAGAGTGAATTCTACCAATGGAAGCCAGGTTAATGATTACAATTAATCTTTTAC - 2160
2161 - TGTACATTCCCAAGGCTTTAGTTTTAAATGCCACTGTGCCTTTAACAAGGTTGTAAATAT - 2220
2221 - TTTATGCCCACCAGAGATGTGGTCATAAGATCTGATCCTGAGCCAGAGATTCAGATGGCA - 2280
2281 - CAGGAAGTATTCATGTATTTTAACACTGGGGTTTTCTTTCTTTCATACTGAGATTTTTTT - 2340
2341 - TCAGTATGTATCCTCCAGCTCTTAAAGCTTACCTGAGAAAGCTTTAAATGAGAAAAGGAC - 2400
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2401 - CATGCGATTGGTGCTGTGTTACATACACATACTTTCTTGGCTTCTGAGTAGCTCAGGTGT - 2460
2461 - GGCTTTTGGCTGCAGATGTTAAATTTTGATACCATGTAAACCTACCCAGCTTCTCAGACT - 2520
2521 - TGGGTCTTGTTTTTTGATGGGAACAGAGGTGTTTAGAGAAAGCCTCTGAGTATGCCTTTC - 2580
2581 - AGATTTTGAACAAGCGGCCTTTTCTAAACATCGACTTCTACTACTCTCTAGCCTTAAAAT - 2640
2641 - ACCTTCTGCTTAGATCCAGGGCCCTTCTACTGGAGATAGGAAAAGTAGAATTCAGGAATT - 2700
2701 - AAAAGAATTACTCTTTATTCAATTTGAGGAACTTGGTGAAAGCCCCTCCTCTTATGACAG - 2760
2761 - CCAGGTTCCTGCTGGCTAGACCAGCCTATTCAGCGCTTTGCTAGGGGATTGGGTGGTCCA - 2820
2821 - CGCACTCGCTAATACAGTTCTCCAGGTGTGGAATGATGTCAATACGATTGCTTGGCCTTT - 2880
2881 - TCCCCCTGTGCCTTTGCTCGGTGCTCTGGTTTCCTCAGCAACACTCCTTGTAAGGGGCAG - 2940
2941 - AGACAGGGTCCACCAACTCCCCCAAGATGAAGAAGCCCCTTTCAGGCCAGTCTGTGGTGG - 3000
3001 - CTCATGCCTGTAACTCCCAGCACTTTGCAAGGCCGAGGAGGGTGGATCACTTGAGGTCAG - 3060
3061 - GAGTTCGAGACCAGCCTGACCAACATGGCGAAACCCCATCTCTACTAAAAATACAAAAAT - 3120
3121 - TAGCTTGGCATGGTGGTGCGTGCCTGTAATCCCAGCTACTCGGGAGGCTGGGGCAGGAGA - 3180
3181 - ATTGCTTGAACTTGGGAGATGGAGGCTGCAGCGAGCCAAGATCGTGCCACTGCACTCCAG - 3240
3241 - CCTGGGCAAGAGTTTTTTTAAGACTCTTAAAAAAAGAGCCTGGGCAATTTTTTTAAGACT - 3300
3301 - CTGTCTTAAAAAAAAAAAAAAAAAAAAAAAAAACTCGACCTGCAGCATGCAAGC - 3353
```

Figure 15. Sequence and translation of the full-length RCP cDNA. The polyA tail is underlined.

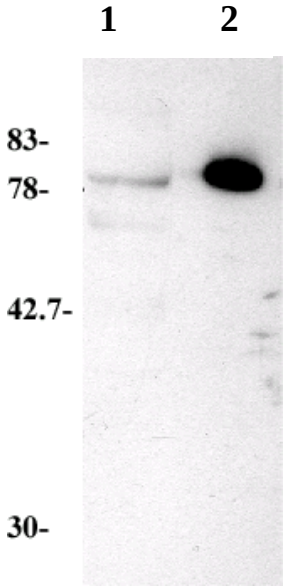


Figure 16. Recombinant RCP migrates at the same molecular weight as endogenous RCP. A postnuclear supernatant from HeLa cells transfected with pGEM1 RCP (lane 2) was run beside a PNS from nontransfected HeLa cells (lane 1), and probed with α-H13 antibody.

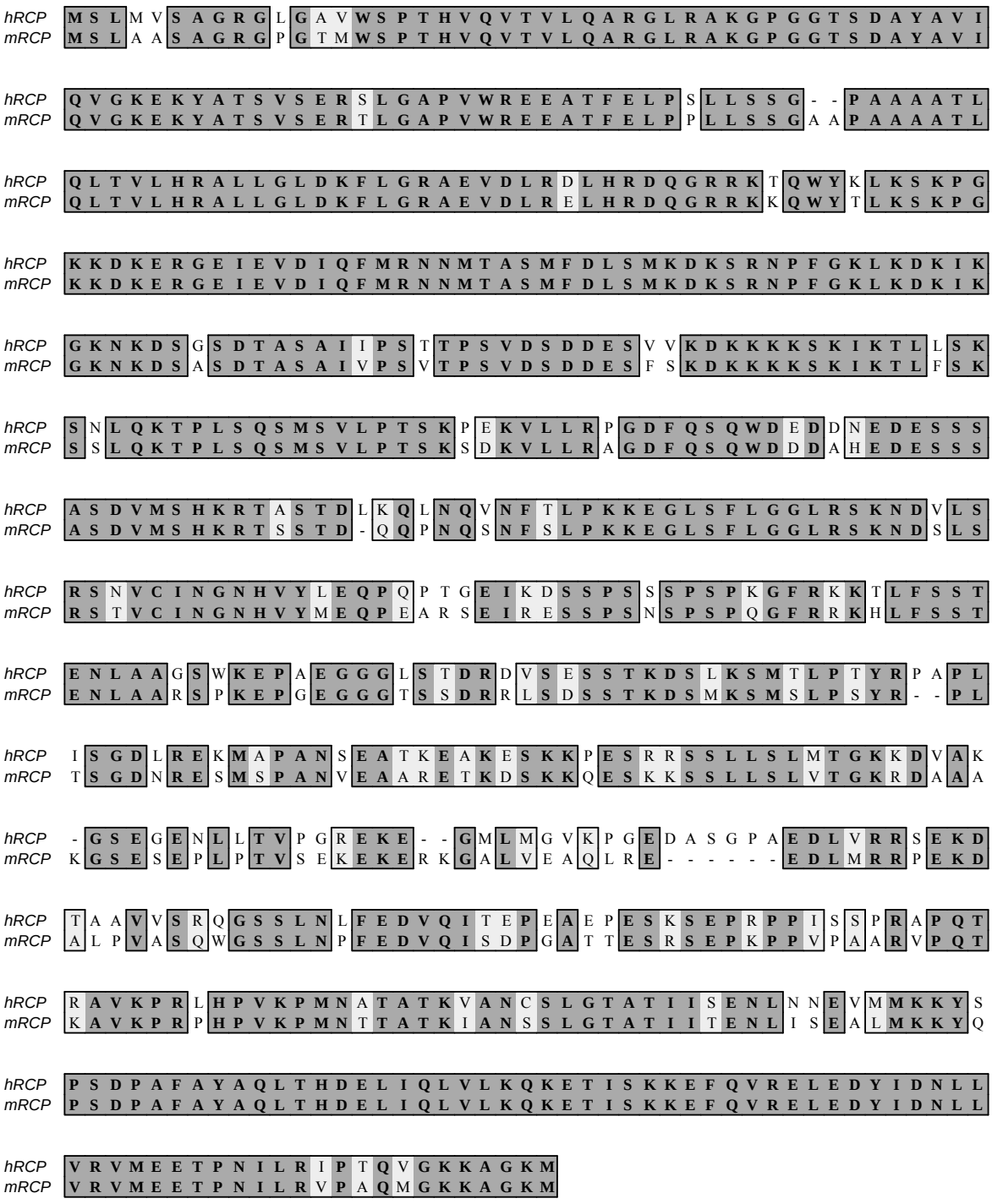


Figure 17. ClustalW alignment of mRCP (AK014696) v. human RCP (hRCP). Identities are highlighted in dark grey, and similarities in light grey.

3.1.6 Analysis of the RCP Amino Acid Sequence

RCP is a 649 amino acid protein, with a predicted molecular weight of 70kDa. The discrepancy between this and the observed molecular weight, of approximately 80kDa, may be due to posttranslational modifications. The RCP primary sequence was used to search a number of databases (Pfam and Prosite). Searching for motifs revealed that it has a C2-domain at its amino-terminus (amino acids 20-122). C2-domains are involved in mediating the binding of proteins to phospholipids. There are two types of C2-domains, calcium-dependent in which binding to phospholipids is regulated by Ca^{2+}, and calcium-independent (Rizo and Sudhof, 1998). Most proteins that contain these domains are involved in either signal transduction e.g. PKC and Ras-GAP, or membrane traffic e.g. Rabphilin-3 and the synaptotagmins, or both. There are a number of putative phosphorylation and glycosylation sites throughout the sequence. Secondary structure analysis revealed that RCP has a high probability of forming two coiled-coil domains, one near the amino-terminus and one at the carboxy-terminus (Lupas, 1997);

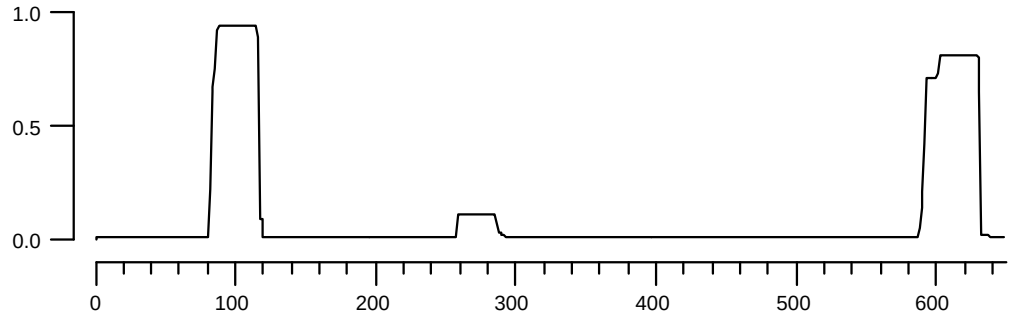


Figure 18. RCP has a high probability of forming two coiled-coil domains. The *x*-axis indicates the amino acid position, and the *y*-axis shows the probability of forming a coiled-coil (0-1).

Coiled-coil domains are bundles of α-helices that wind into a superhelix. They are involved in protein-protein interactions. Coiled-coils are also a common feature in proteins that form homodimers.

Using the RCP amino acid sequence in a 'BLAST' search of the GenBank non-redundant database revealed that it has two close human homologues, KIAA0857 and KIAA0941 (Figure 19). The cDNAs for these two proteins were cloned at the

Kazusa DNA Research Institute as part of a project to clone unidentified human cDNAs with the potential of coding for large proteins (Nagase et al., 1998). These two proteins shared a high degree of homology with RCP at their amino- and carboxy-termini. They both contained an amino-terminal C2 domain, and a carboxy-terminal coiled-coil domain. KIAA0857 has since been reported in the literature as pp75, a phosphoprotein that may be involved in lupus erythematosus (Wang *et al.,* 1999). A subsequent independent study identified KIAA0857 as a Rab11 interacting protein involved in the regulation of apical membrane trafficking in polarised epithelial cells, and renamed it Rip11 (Prekeris *et al.,* 2000). Work on KIAA0941 has revealed that it also interacts with Rab11 (see Section 3.2). These three proteins are, therefore, members of a family of Rab11 interacting proteins. RCP is unique in that it also interacts with Rab4. There are a number of more distant relatives (see Discussion), including KIAA0665 (Hales *et al.,* 2001; Prekeris *et al.,* 2001) and KIAA1821 (personal communication, Alan Hendrick & Deborah Wallace), both of which also interact with Rab11 but not with Rab4. *Drosophila melanogaster* has one member of this family, CG6066, which appears to be the progenitor of RCP, Rip11, and KIAA0941. The *Drosophila* phosphoprotein, Nuf1, may be the progenitor of KIAA0665 and KIAA1821 (Rothwell *et al.,* 1998).

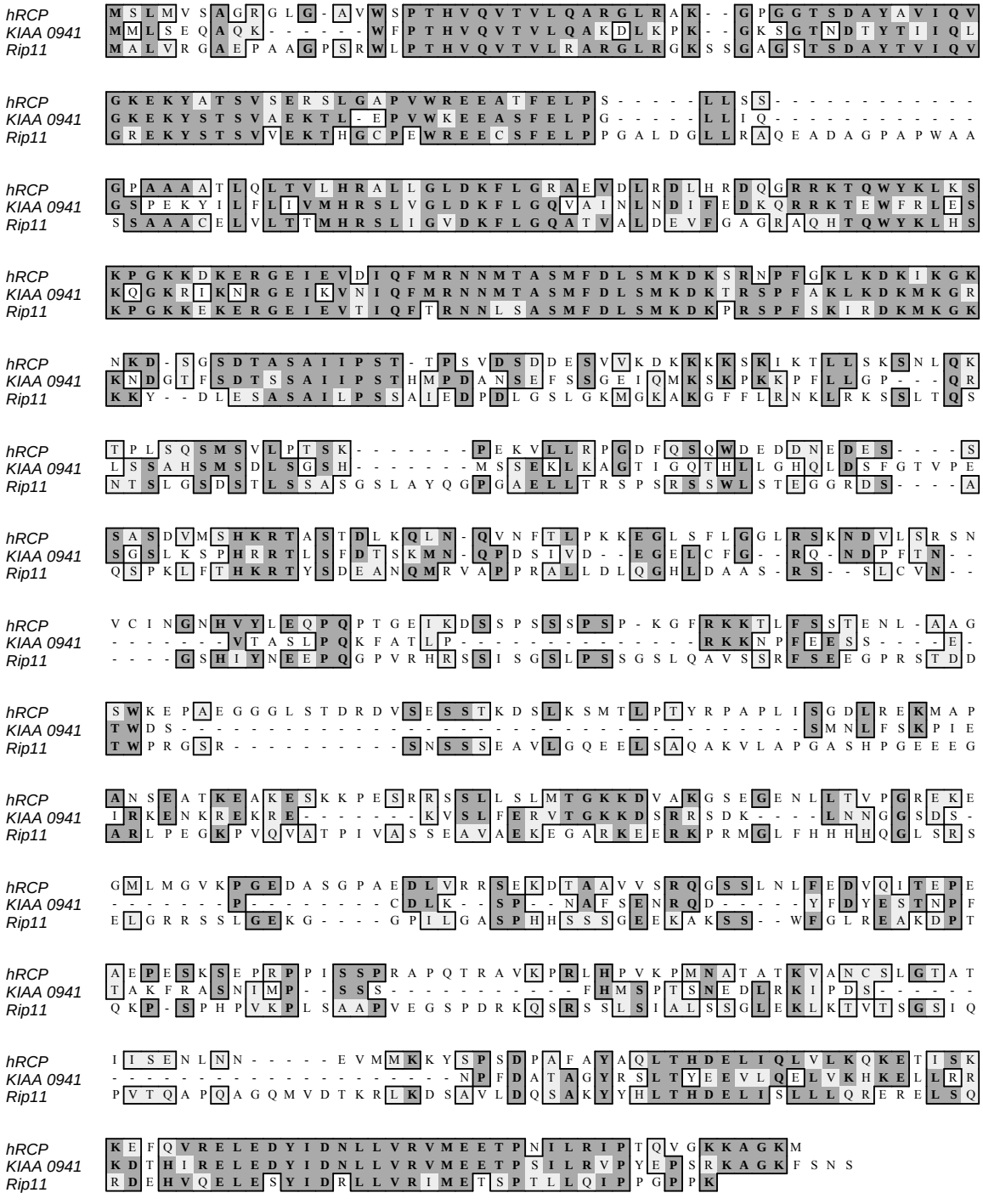


Figure 19. ClustalW alignment of hRCP, KIAA0941, and Rip11. Identities are highlighted in dark grey and similarities are in light grey.

This alignment reveals that the three human members of this family share highly homologous amino-termini, which encompass their C2-domains. Their carboxy-termini also show significant homology, they all have the potential to form a coiled-coil. Yeast two-hybrid analysis has shown that the carboxy-terminus mediates the interaction with their Rab partner/s. These two domains are separated by a less conserved, intervening region. It is likely that the two conserved domains are important in mediating the RCP-family cellular functions.

To further analyse the expression pattern of RCP, a gene expression panel (Origene) was analysed. The expression panel consisted of the first strand cDNA from various human tissues. These cDNAs were used as the template in PCR reactions with internal RCP gene-specific primers (GSP-1 and GSP-2). If RCP is expressed in the tissues a band of the appropriate size will be seen when the PCR is separated on an agarose gel;

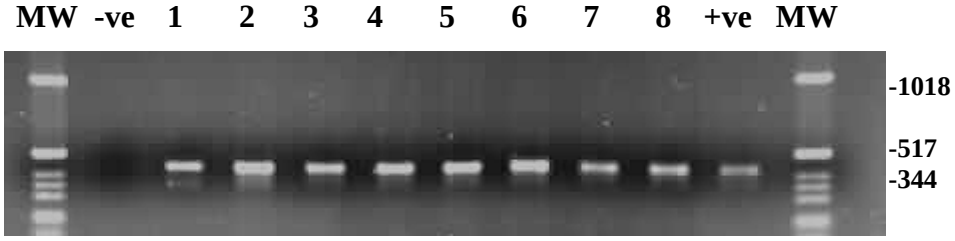


Figure 20. PCR analysis of a human gene expression panel with RCP gene-specific primers. MW. size markers; -ve. negative control 1. brain; 2. heart; 3. testis; 4. lung; 5. spleen; 6. ovary; 7. small intestine; 8. liver; +ve. positive control.

A 435bp product was amplified in all the tissues tested, which corresponds with the product amplified when the clone H13 plasmid was used as template, in the positive control reaction. The negative control contained a complete PCR reaction, but lacked template.

3.1.7 RCP is a Predominantly Membrane-Bound Protein

Since Rab proteins cycle between membranes and the cytosol, the RCP/Rab interaction may occur in the cytosol, or on endosomes. To determine the subcellular localisation of RCP, postnuclear supernatant, a cytoslic fraction (100,000g supernatant), and a membrane fraction (100,000g pellet), from HeLa cells was probed

with the affinity-purified H13p antibody. RCP was found predominantly in the membrane fraction;

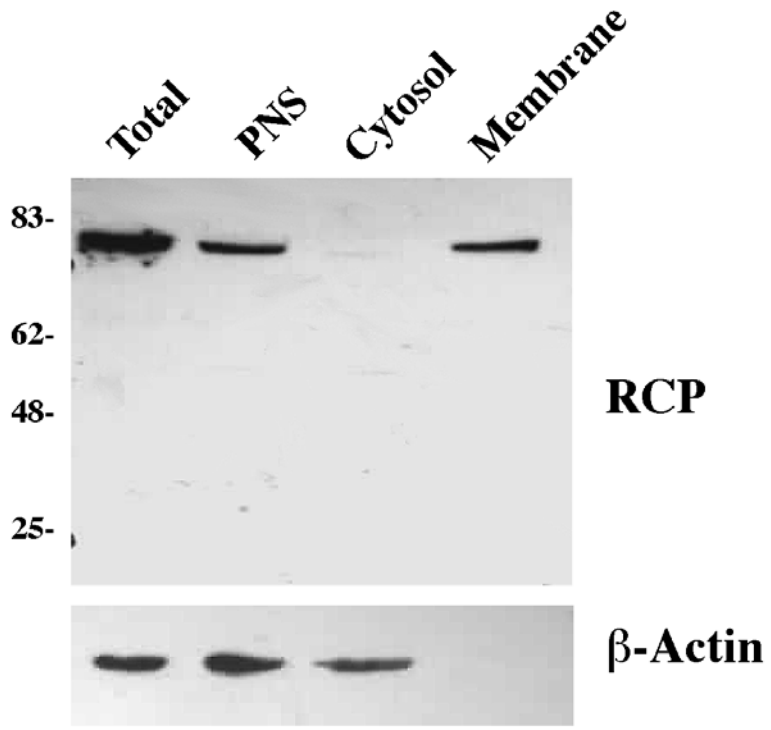


Figure 21. RCP is a membrane bound protein. Western blot using H13p antibody to probe a total protein fraction, PNS, cytosol, and membrane extracts from HeLa cells.

When this initial fractionation was performed, no other Rab effector proteins had been identified that were predominantly membrane-bound. The conventional idea of a Rab effector is of a cytosolic protein that is recruited to the membranes via its activated Rab. Rabaptin-5 and Rabphilin-3 are examples of such proteins (Shirataki *et al.,* 1993; Stenmark *et al.,* 1995). However, recently a number of membrane-bound Rab interacting proteins have been identified, including Rab5ip, a membrane-bound Rab5 binding protein that has an N-terminal transmembrane domain (Hoffenberg *et al.,* 2000). Rip11, a RCP homologue, is also predominantly membrane-bound (Prekeris *et al.,* 2000), as is the FYVE domain containing Rab4 interacting protein, Rabip4 (Cormont *et al.,* 2001).

No putative transmembrane domains were identified in the RCP amino acid sequence, suggesting that it is a peripheral membrane protein. To determine the stability of RCP on membranes, HeLa postnuclear supernatants were treated with high

salt concentrations, high pH, or detergent, prior to separation into cytosolic and membrane fractions;

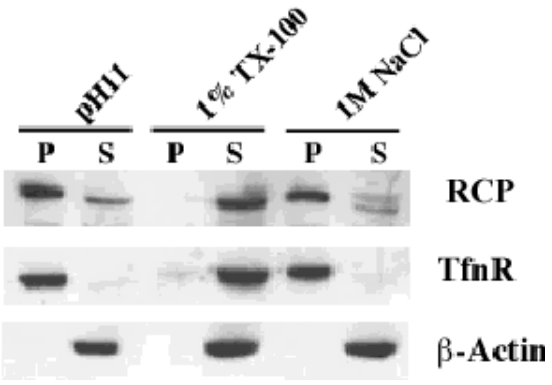


Figure 22. Fractionation of HeLa cells. The PNS of HeLa cells were incubated with high pH, 1M NaCl, or 1% TX-100 prior to separation into 100,000g pellet (P) and 100,000g supernatant (S) fractions. The fractions were probed with affinity purified H13p antibody. The accuracy of the fractionation procedure was confirmed by probing for the TfnR (an integral membrane protein), and β-actin (a cytosolic protein).

The majority of RCP appeared to be resistant to extraction by 1M NaCl and high pH, however, RCP was completely soluble in 1% Triton X-100. This contrasts somewhat with the membrane stability of Rip11. Rip11 is resistant to high salt concentrations, but is partially extracted by high pH, and only approximately 50% is extracted from the membrane by 1% TX-100. These differences may reflect the different roles that these two proteins play. Unlike RCP, Rip11 displays a preference for GTP-bound Rab11 and Prekeris *et al.* suggest that Rip11 binds to the recycling compartment membrane via Rab11 and by interaction with neutral phospholipids.

3.1.8 H13p Interacts with Rab4 and Rab11 in vitro

The H13p interaction with Rab4 and Rab11 in the yeast two-hybrid system appears to be specific, in that it does not interact with other, closely related, Rab GTPases. Also, the interaction with Rab4 is nucleotide-dependent. However, it is necessary to demonstrate the interaction in other systems. To show a direct interaction between H13p and Rab4, or Rab11, an *in vitro* overlay assay was used. GST-tagged

H13p was transferred to nitrocellulose and incubated with [^{32}P]GTP-labelled Rab4 or Rab11. After incubation with the radioloabelled Rab protein, the nitrocellulose was washed and exposed on a phosphoimager;

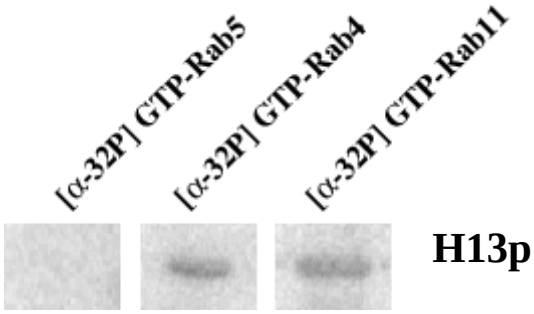


Figure 23. *In vitro* interaction between H13p and radiolabelled Rab4 or Rab11. Radiolabelled Rab5 was used as a negative control.

Both Rab4 and Rab11 bound to H13p on the nitrocellulose, as indicated by a band at 37kDa. As a negative control [^{32}P]GTP-Rab5 was used. As expected, Rab5 did not show any interaction with H13p. These data demonstrated that the interactions were not an artefact of the two-hybrid system and that no accessory factors were necessary i.e. purified H13p can interact with purified Rab4 or Rab11.

3.1.9 RCP displays a Punctate Distribution in HeLa Cells and Colocalises with Rab4, Rab11, and Markers of the Recycling Pathway

All the evidence presented so far suggests that RCP is a genuine Rab4 and Rab11 interacting protein. If this is the case, then RCP should localise to part, or all, of the recycling pathway in HeLa cells, since both of its Rab partners play roles in regulating transport along this pathway. HeLa cells were chosen for most of the cell culture applications since they are easy to culture, they have a well defined recycling pathway, they are relatively easy to transfect, and clone H13 was identified from a HeLa cDNA library. The transferrin receptor and its ligand, transferrin, are excellent markers of the recycling pathway. Iron-saturated transferrin binds to the TfnR at the cell surface. The Tfn/TfnR complex is internalised in clathrin-coated vesicles and

delivered to the early sorting endosome, where the low pH causes the dissociation of the iron. The Tfn/TfnR complex is then recycled back to the plasma membrane, either directly from the sorting endosome, or in a 'slow' cycle via the perinuclear recycling compartment.

3.1.9.1 Distribution of RCP in HeLa Cells

To determine the localisation of RCP within the cell, the affinity-purified H13 antibody was tested for its ability to detect the endogenous protein in HeLa cells, by immunofluorescence. Various dilutions of the antibody were tested and it was determined that a 1/100 dilution gave the best signal. RCP displays a vesicular pattern throughout the cytoplasm, but with a concentration of the vesicles in the perinuclear region. To determine if RCP colocalises with the TfnR, HeLa cells were colabelled with the H13p-antibody and a monoclonal TfnR antibody. The TfnR pattern in HeLa cells is vesicular. These vesicles appear to be either large with a globular appearance, or finer reminiscent of the RCP-vesicles. RCP and the TfnR colocalise in the perinulear region (Figure 24A). However, there are also clearly vesicles that are either RCP-positive or TfnR-positive. Since the TfnR is found throughout the early endosomal pathway, which includes the plasma membrane, CCVs, uncoated vesicles, the sorting endosmes, and the recycling compartment, it is possible that RCP and the TfnR colocalise on only one of these compartments. RCP does not colocalise with lysobisphosphatidic acid (LBPA), a phospholipid found in late endosomes and lysosomes (Figure 24D), and thus a marker of the late endocytic pathway.

3.1.9.2 RCP Colocalises with Rab4-GFP

The task of colocalising RCP with Rab4 is complicated by the lack of available antibodies that can detect endogenous Rab4, by immunofluorescence. To overcome this problem, a HeLa cell line that stably expresses Rab4WT with an amino-terminal green fluorescent protein (GFP) fusion was used (Bielli *et al.,* 2001). GFP-Rab4WT labels large vesicles in the perinuclear area. Labelling these cells with H13p-antibody revealed partial colocalistion between RCP and Rab4 (Figure 24B).

The majority of Rab4 is found on the early sorting endosome, however, a number of recent reports have shown that a fraction of Rab4 overlaps with Rab11 on the recycling compartment (Sonnichsen *et al.,* 2000; Trischler *et al.,* 1999). It is possible that the RCP colocalisation with Rab4 is at the recycling compatment.

3.1.9.3 RCP Colocalises with Rab11

To investigate the colocalisation with respect to Rab11, an affinity-purified rabbit Rab11 antibody was used, that detects the endogenous Rab11. Since the H13p-antibody was also raised in rabbits it was necessary to express exogenous RCP and use a monoclonal antibody to an amino-terminal epitope. δRCP was subcloned into the pcDNA3.1HisB vector and expressed in HeLa cells from the CMV promoter present on the vector. A time-course experiment showed that transfecting for between 6 and 8 hours resulted in a δRCP pattern that was similar to that of endogenous RCP. When δRCP expressing cells were colabelled with the Rab11 antibody, extensive colocalisation was seen between the two proteins. In fact, when the Rab11 pattern was compared between cells expressing δRCP, and nontransfected cells it appeared that Rab11 was recruited, by the recombinant protein, from the cytosol to the membranes (Figure 24C). In some of the transfected cells, δRCP could be seen on tubular structures, with Rab11 also labelling these tubules. No similar recruitment of Rab4 was seen under the same conditions.

3.1.9.4 Summary

A number of conclusions can be drawn from these colocalisation experiments. The observation that RCP only partially colocalises with the TfnR and Rab4 suggests that it is not found throughout the early endosomal pathway, but at a more restricted location along this pathway. This location is probably the recycling compartment since RCP shows almost total colocalisation with Rab11. However, since Rab11 has also been seen on the TGN it is possible that there is also some RCP on this organelle. It appears that RCP recruits Rab11 from the cytosol to the membrane. This may explain the RCP/Rab11 interaction profile from the yeast two-hybrid system. Since

cytosolic Rab proteins are GDP-bound, if RCP was to recruit Rab11 from the cytosol, it would need to have the ability to interact with the inactive conformation of Rab11. Since there was no similar recruitment of Rab4 seen, it can be postulated that RCP interacts with Rab4 that is already membrane-bound, and it serves to recruit Rab11, from the cytosol to the correct intracellular compartment. In this way RCP may act as the intermediary between Rab4-regulated transport and Rab11-regulated vesicular transport.

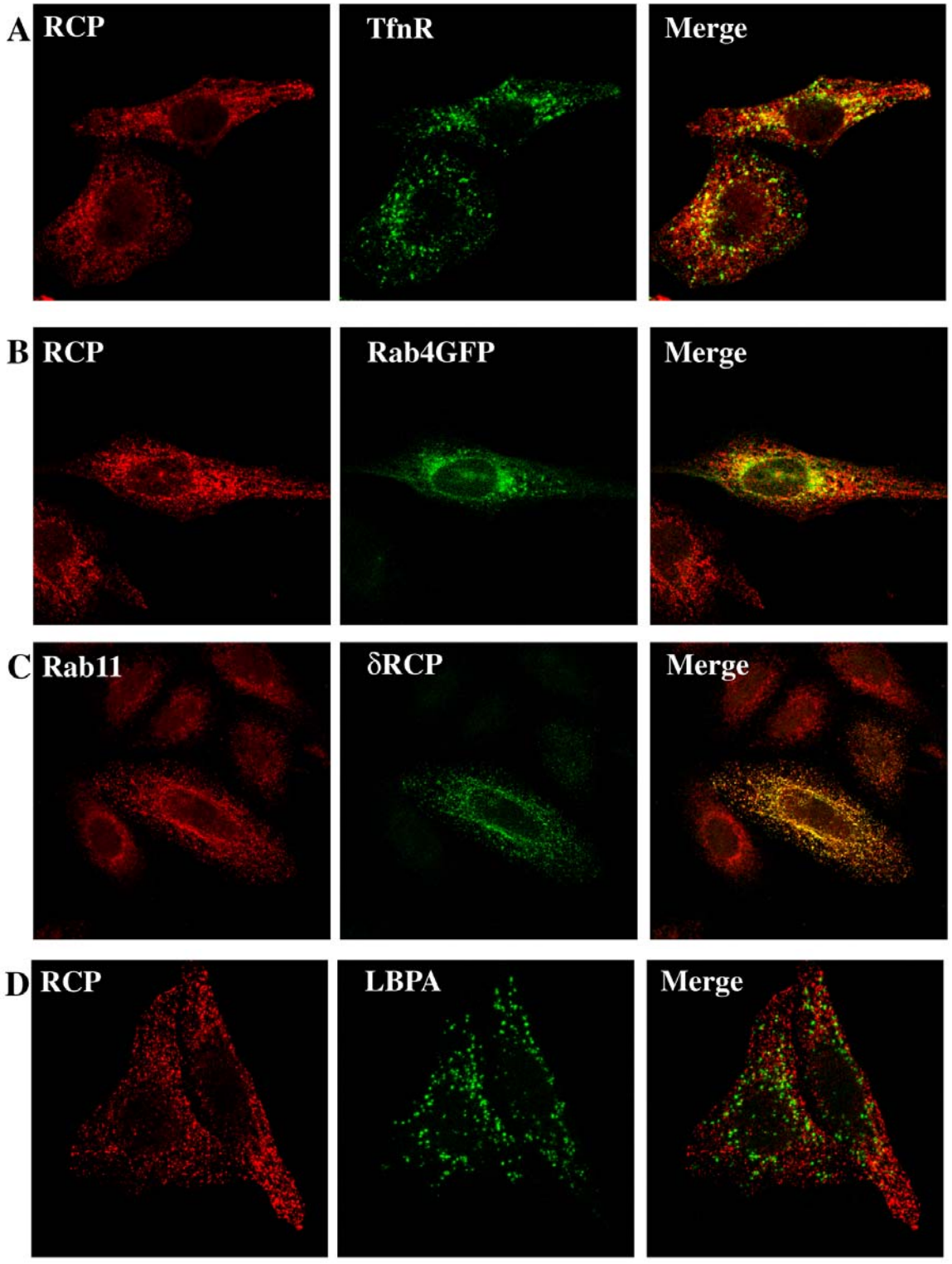


Figure 24. RCP localises to the receptor recycling pathway in HeLa cells. Confocal images of HeLa cells labelled with anti-H13 (red) and with anti-TfnR (green) **(A)**, Rab4GFP (green) expressing cells labelled with anti-H13 (red) **(B)**, δRCP was overexpressed and detected with anti-Xpress (green) and co-labelled with anti-Rab11 (red) **(C)**, and anti-H13 (red) and anti-LBPA (green) **(D)**.

3.1.10 Overexpression of H13p in HeLa Cells Results in the Formation of a Tubular FITC-Transferrin Positive Membrane Network

If RCP functions along the receptor recycling pathway then expression of H13p, which contains the Rab4 and Rab11 interacting domain of RCP, in cultured cells should have an effect on the recycling of ligands. In the following series of experiments, H13p, or various Rab constructs, are overexpressed in HeLa cells and analysed for any effect they may have on the uptake and recycling of fluorescently labelled iron-saturated Tfn. This technique has been used extensively to analyse proteins involved in endocytosis (McCaffrey *et al.,* 2001; Stenmark *et al.,* 1995; Ullrich *et al.,* 1996; van der Sluijs *et al.,* 2001; Vitale *et al.,* 1998; Wilcke *et al.,* 2000).

To overexpress H13p, the H13 reading frame was subcloned into the mammalian expression vector pcDNA3.1HisB (InVitrogen). In this vector expression of the insert can be driven by either a cytomegalovirus (CMV) promoter, or from a T7 promoter. For these experiments the T7 promoter was used in conjunction with an attenuated form of the vaccinia virus. The virus, once infected into mammalian cells, expresses the T7 RNA polymerase. The advantage of this system is its ability to express high levels of protein in a short time (3-4 hours post-transfection).

3.1.10.1 FITC-Tfn Uptake at 37^{o}C

HeLa cells, grown on glass coverslips, were infected with the modified vaccinia virus and then transfected with either pcDNA3.1HisB H13, or the empty vector as a control. The transfection was allowed to proceed for 6 hours, during which time high levels of H13p are expressed. After transfection the cells were allowed to endocytose FITC-Tfn for 45 minutes at 37^{o}C. The cells were immediately washed with ice-cold 1X PBS and fixed. Transfected cells were identified with the α-H13 antibody, or a monoclonal antibody against the amino-terminal Xpress epitope. In the control cells the FITC-Tfn was found in punctate vesicles throughout the cytoplasm (Figure 25A), with an accumulation at the perinuclear region. In cells expressing H13p the morphology of the Tfn compartment was dramatically changed. Instead of a vesicular pattern, the FITC-Tfn was found in an intricate tubular network that

extended from the perinuclear region (Figure 25B). Since this pattern was never seen in control cells the effect can be attributed to the expression of H13p. The tubules were seen in some cells after 4 hours, with the majority of transfected cells developing them after 5 to 6 hours. Since the FITC-Tfn was able to enter the cell and label these tubules it can be concluded that overexpression of H13p does not inhibit the uptake of transferrin.

What are these tubules, and how do they develop? The recycling compartment, when visualised by EM and by immunofluorescent techniques, has been described as a tubulo-vesicular network (Sonnichsen *et al.,* 2000; Trischler *et al.,* 1999). It is possible that these H13p-induced tubules are due to an enlargement of the recycling compartment, as opposed to the creation of an abnormal compartment. The formation of these tubules may be explained in two ways; (i) overexpression of H13p results in an increased rate of transport into the recycling compartment, and without a corresponding increase in the rate of trafficking out, transport vesicles accumulate in the recycling compartment resulting in its enlargement, or (ii) overexpression of H13p results in an inhibition of trafficking from the recycling compartment back to the plasma membrane, or to the TGN. With transport vesicles entering the recycling compartment, but none exiting, the compartment grows into the tubular structure observed.

An unexpected result from these experiments was observed in a small proportion of transfected cells. In these cells the level of H13p expression was low, such that the cytoplasm was not filled with the recombinant protein, yet tubule formation was still induced. H13p could be seen on these tubules perfectly colocalising with the FITC-Tfn (Figure 25C). Since H13p lacks the C2-domain of RCP it can be concluded that this domain is not required for the membrane localisation of RCP. However, the C2 domain is likely to have some role in RCP membrane binding, such as in the correct sub-compartment localisation of the protein (see Discussion), or for providing a means of regulating its membrane association/dissociation. The H13 reading frame does not have any hydrophobic regions or other known membrane binding motifs, therefore, it is possible that RCP localises to the membranes by binding to an integral membrane protein in conjunction with a direct interaction with the membrane phospholipids. It cannot be ruled out that RCP interacts indirectly, via Rab4 or Rab11, with the recycling compartment membrane. However, this is improbable since it would be unlikely that there would

sufficient levels of endogenous membrane-bound Rab4, or Rab11, to bind the high quantities of H13p expressed by the vaccinia system. The RCP amino terminus is likely to have a role(s) in mediating the Rab4 and/or Rab11 downstream signals, since its absence results in such a dramatic phenotype.

3.1.10.2 FITC-Tfn Uptake at 18°C

Since RCP interacts with both Rab4, a predominantly early sorting endosome protein, and, Rab11, a recycling compartment protein, the H13p-induced tubules may be the result of an enlargement of one or other of the two compartments. Alternatively, they may be due to the two compartments fusing together. A minor pool of Rab11 is also found on the TGN, however, it is unlikely that the tubules are part of this compartment since they are accessible to Tfn. The sorting endosome and the recycling compartment have been described in some cell lines as being part of a continuous tubular network (Sonnichsen *et al.,* 2000). However, they can be distinguished in a number of ways, (i) cargo can access the sorting endosome at 18°C but will not progress to the recycling compartment (Wilcke *et al.,* 2000), and (ii) EGF will enter the sorting endosome on its way to the lysosomes, but only a minor fraction will proceed along the recycling pathway.

In order to determine the origin of the H13p-induced tubules, FITC-Tfn was allowed to endocytose into H13 transfected, or mock transfected, cells at 18°C. If the tubules are formed from the early sorting endosome then they should be accessible to FITC-Tfn under these conditions. Conversely, if the tubules are part of the recycling compartment, then the FITC-Tfn should not be able to enter them at this temperature. In the mock transfected cells, where uptake was performed at 18°C for 45 minutes and after which the cells were immediately fixed, FITC-Tfn showed a vesicular pattern in the periphery of the cell, with no concentration in the perinuclear region. A similar pattern was seen in cells expressing H13p. In these cells no FITC-Tfn was observed in tubular structures. However, if the FITC-Tfn was internalised at 18°C for 45 minutes and then chased at 37°C for 10 minutes, prior to fixation, the ligand was able to access the H13p-induced tubules (Figure 26). Similar results were seen in cells expressing Rab11S25N, which also induces tubulation of the FITC-Tfn compartment (data not shown).

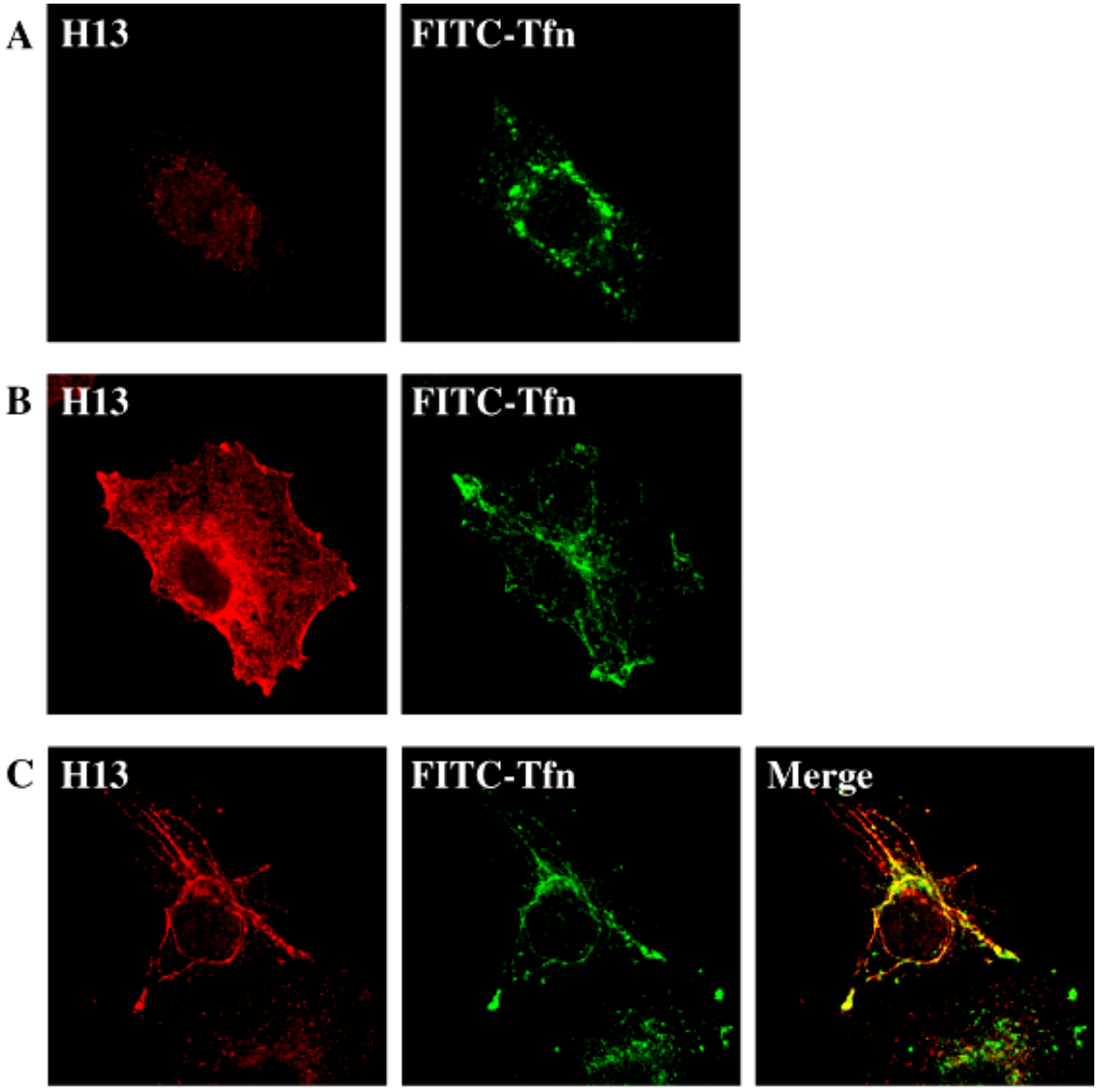


Figure 25. Overexpression of H13p causes a perturbation of the transferrin compartment. FITC-Tfn (green) was continuously internalised at 37^{o}C in mock transfected cells **(A)**, in cells overexpressing H13p (red) **(B)**, and cells minimally expressing H13p (red) **(C)**.

3.1.10.3 Internalisation of Texas Red-EGF

To further demonstrate that the H13p-induced tubules are derived from the recycling compartment, transfected cells were allowed to co-internalise FITC-Tfn and Texas Red (TxR)-EGF, at 37^{o}C for 1 hour. The majority of epidermal growth factor (EGF) follows the late endocytic pathway (Renfrew and Hubbard, 1991). Once bound to its receptor at the cell surface, the complex is transported to the sorting endosomes in CCVs. From the sorting endosomes the EGF/EGF receptor complex is transported to the late endosomes and is eventually degraded in lysosomes. In cells that expressed H13p, Rab11S25N (Wilcke *et al.,* 2000), or Rab4Q67L (McCaffrey *et al.,* 2001), FITC-Tfn was found in the tubular structures, whereas the TxR-EGF was found in several large vesicular structures within the cell, where it colocalised with FITC-Tfn. No TxR-EGF was observed labelling the extent of the tubules (Figure 27).

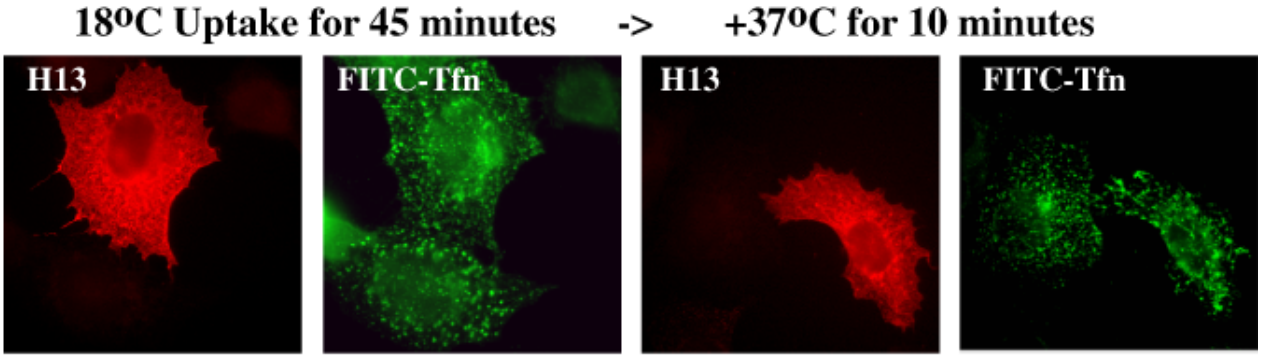


Figure 26. FITC-Tfn does not enter H13p-induced tubules at 18°C. FITC-Tfn was internalised into H13p-expressing cells for 45 minutes at 18°C and either immediately fixed, or chased at 37°C for 10 minutes, prior to fixation.

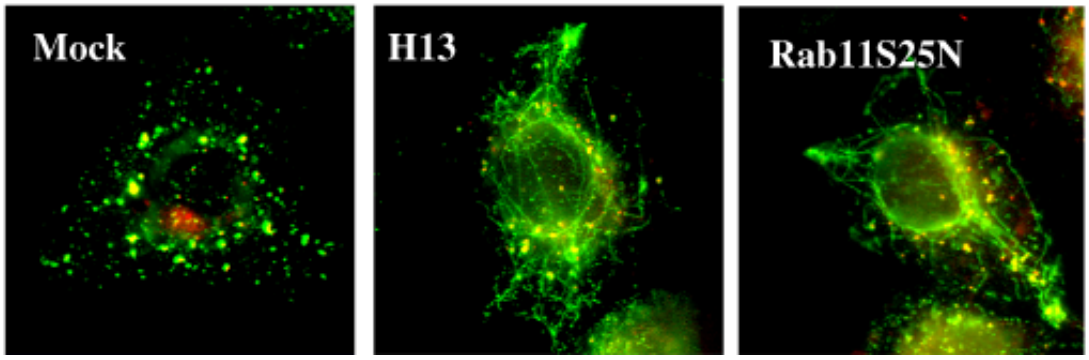


Figure 27. EGF does not enter the H13p-induced tubules. FITC-Tfn (green) and TxR-EGF (red) were co-internalised at 37°C for 1 hour in HeLa cells transfected with pcDNA3.1HisB (Mock), pcDNA3.1HisB H13 (H13), or pGEM1 Rab11S25N (Rab11S25N).

3.1.10.4 Summary

A powerful technique for determining the function of a protein is to overexpress the protein of interest, or a mutant form, in cells and determine its effect on an *in vivo* assay. In this case H13p, a truncated mutant of RCP, was overexpressed in HeLa cells and tested for an effect on the intracellular transport of the fluorescently labelled ligands, FITC-Tfn and TxR-EGF. Transferrin follows the receptor recycling pathway, it enters the cell in a clathrin coated vesicle and is transported to the early sorting endosome. After discharging its cargo of iron it is recycled back to the plasma membrane, either directly from the sorting endosome, or in a 'slow' cycle via the perinuclear recycling compartment. EGF, when bound to its receptor on the cell surface, follows the same pathway as Tfn to the early sorting endosome. But from here the majority of EGF is sorted for transport along the late endocytic pathway, where it eventually ends up in lysosomes for degradation. Thus, these two ligands can be used to determine any qualitative effect H13p may have on the recycling or degradative pathways.

Rab4 is located at the 'junction' of these pathways, the early sorting endosome, and plays a role in regulating transport along both of these routes (McCaffrey *et al.,* 2001). Therefore, it was considered possible that RCP may have a role in one, or both, pathways. However, two pieces of evidence suggested RCP is involved only in the recycling pathway, (i) in addition to interacting with Rab4, it also interacts with Rab11, a recycling compartment protein, and (ii) in the cell it shows greater colocalisation with Rab11 than with Rab4, suggesting that it is found on the recycling compartment. There is only minor colocalisation with EEA1, a marker for the sorting endosome. It is possible RCP interacts only with the pool of Rab4 that travels to the recycling compartment. Since it was considered more likely that RCP functions along the recycling pathway, the effect of H13p-overexpression on the trafficking of FITC-Tfn was analysed in detail.

H13p-overexpression results in the formation of an intricate tubular network that can be labelled with FITC-Tfn. To elucidate whether these tubules are derived from the early sorting endosomes or the recycling compartment, or are an amalgamation of both, two approaches were taken. The trafficking of FITC-Tfn at 18^{o}C revealed that the H13p-induced tubules were inaccessible to the ligand at this temperature (Figure 26). Transport from the sorting endosomes to the recycling

compartment is inhibited at 18°C (Ullrich *et al.,* 1996; Wilcke *et al.,* 2000). However, after a short chase at 37°C the FITC-Tfn was clearly found in tubules. The other approach was to follow the transport of EGF. The majority of EGF does not enter the recycling compartment but is sent along the late endocytic pathway for degradation. If the H13p-induced tubules were an abnormal/enlarged sorting endosome, then it would be likely that the TxR-EGF would label them. However, EGF was not seen in a tubular structure, but rather, the ligand was associated with large vesicular structures, overlapping, or near, the tubules (Figure 27).

The most plausible explanation of the results presented in this section is that H13p, which contains the Rab4 and Rab11 binding domain of RCP, locks the two GTPases in a nonfunctional complex. Without the presence of the amino-terminus, H13p cannot perform the normal RCP function upon Rab binding. Since both GTPases regulate steps in the recycling pathway, transport along this pathway is inhibited. With transport vesicles entering the recycling compartment, but the downstream, Rab11-regulated, trafficking out of the recycling compartment inhibited, the result is the enlargement of this compartment, seen as the FITC-Tfn labelled tubular network.

3.1.11 Expression of Active Rab11 Rescues the H13p Phenotype

Rab4 and Rab11 function sequentially in the recycling pathway i.e. transport vesicles derived from the sorting endosome, that are destined for the 'slow' recycling pathway, are under Rab4 regulation until they are delivered to the recycling compartment, where they come under the control of Rab11. If RCP acts as the intermediary between Rab4-regulated transport vesicles and Rab11-regulated transport, then RCP would function downstream of Rab4 and upstream of Rab11 in the receptor recycling pathway. Due to the sequestration of endogenous Rab11 in an inactive complex, the GTPase is not able to perform its downstream function. If this is the case, then it would be expected that addition of Rab11WT or its constitutively active mutant, Rab11Q70L, to cells expressing H13p would reverse the tubulation (see Figure 28). To test this hypothesis, FITC-Tfn was continuously internalised at 37°C in cells cotransfected with clone H13 and Rab11WT or Rab11Q70L. In cells transfected with clone H13 and the pGEM1 empty vector extensive tubulation was

observed, as expected (Figure 29A). However, in cells expressing H13p and Rab11WT or Rab11Q70L no tubulation was observed (Figure 29B&C).

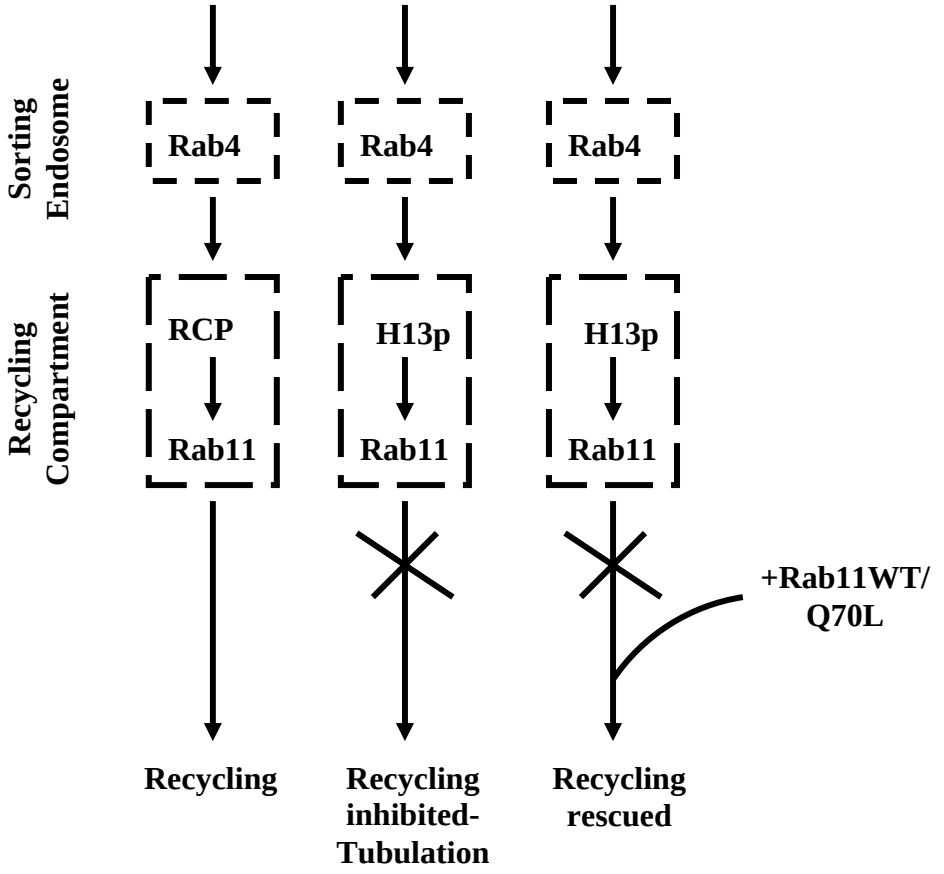


Figure 28. Schematic representation of the proposed Rab4/RCP/Rab11 network, with the effects of H13p addition with/without active Rab11. Arrows indicate the direction of transport.

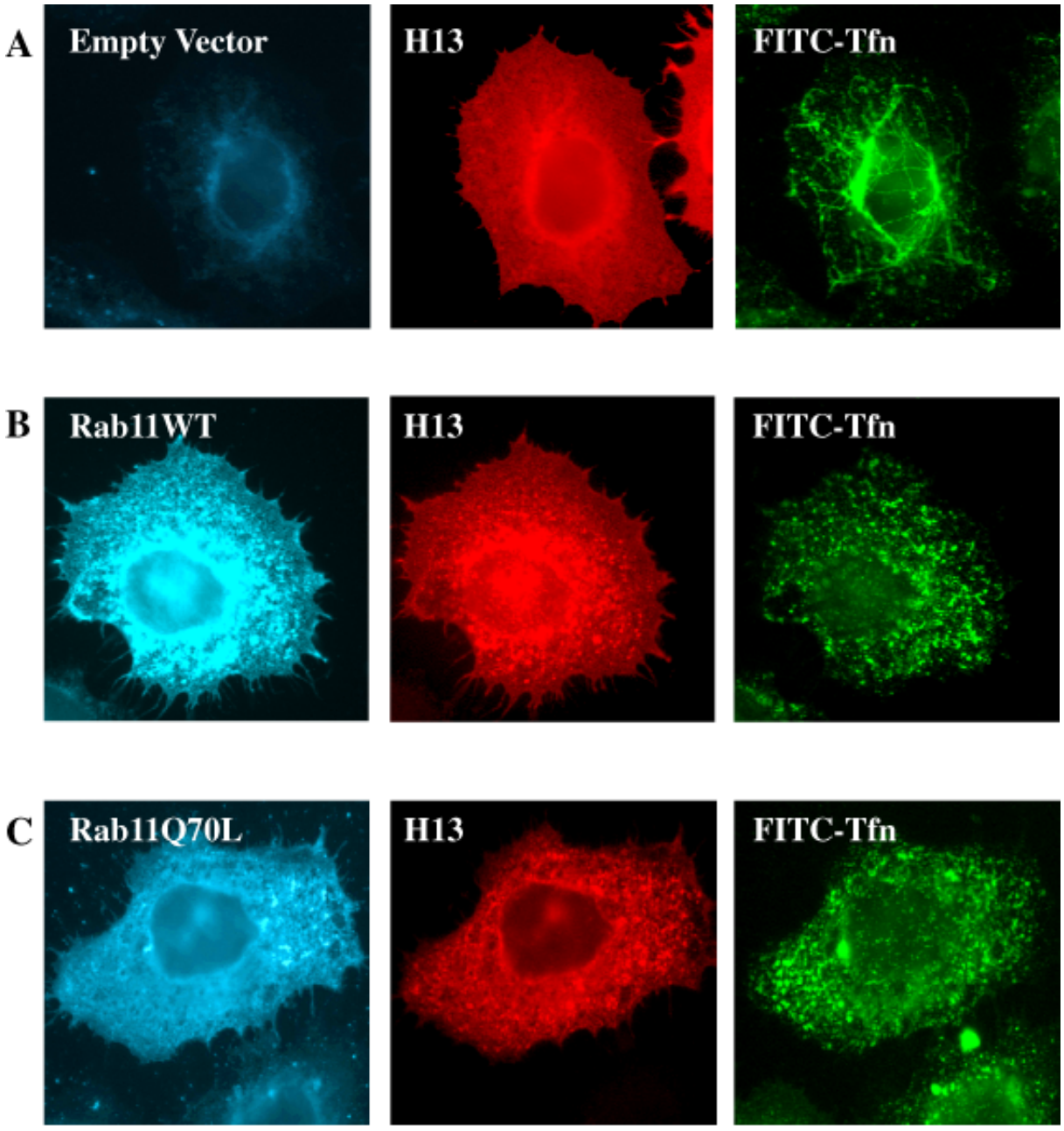


Figure 29. Active Rab11 reverses the H13p phenotype. FITC-Tfn was internalised at 37^{o}C in HeLa cells expressing H13p (red) alone **(A)**, Rab11WT (cyan) and H13p (red) **(B)**, or Rab11Q70L and H13p **(C)**.

These results are consistent with the hypothesis that Rab11 functions downstream of RCP. The inhibition of recycling that results from the overexpression of H13p is bypassed by the addition of active Rab11, which compensates for the endogenous Rab11 that is sequestered in an inactive complex with H13p. This also explains why H13p expression gives a phenotype similar to the GDP-locked mutant of Rab11, since in both cases Rab11 cannot perform its downstream function.

3.1.12 In Vitro *Recycling Assay*

To quantitatively analyse the effect that overexpression of H13p, or full-length RCP, has on the recycling of Tfn, an *in vitro* assay that measures the uptake and recycling of ^{125}I-Tfn was employed (these experiments were kindly performed by Dr Cecilia Bucci, University of Naples, Italy). BHK cells were co-transfected with pcDNA3.1HisB H13, or pGEM1 RCP, and a vector expressing the human TfnR. Expression was driven by T7 RNA polymerase from the vaccinia system. ^{125}I-Tfn was bound to the surface of the cell at 4^{o}C, excess ligand was washed off, and the bound Tfn was allowed to internalise. The amount of ^{125}I-Tfn recycled out of the cell was measured at different time points, and compared to the amount of recycled Tfn in control cells, i.e. cells transfected with the human TfnR alone (Figure 30A). In cells expressing H13p there is almost 50% less ^{125}I-Tfn in the culture medium at time-points from 15 minutes on, when compared to the control cells, suggesting that H13p is dramatically inhibiting the recycling of Tfn. In contrast, full-length RCP appears to slightly stimulate recycling. The effect on recycling of dominant negative Rab4, Rab4S22N, was also tested and compared with that of H13p. H13p showed a more significant inhibition of ^{125}I-Tfn recycling.

Due to the localisation of RCP on the recycling compartment it was decided to analyse the effect that H13p, or full-length RCP, has on the rate of recycling specifically along the 'slow' pathway. To preferentially quantitate traffic through the recycling compartment, transfected BHK cells were pulsed with ^{125}I-Tfn for 5 minutes, excess ligand was washed off, and the cells were incubated for a further 30 minutes at 37^{o}C to allow the ^{125}I-Tfn following the 'fast' pathway to chase out of the cell, and the Tfn trafficking along the 'slow' pathway to enter the recycling compartment. Recycling from this compartment was then measured (Ullrich *et al.*,

1996). Again overexpression of H13p resulted in a strong inhibition of ^{125}I-Tfn recycling. Full-length RCP stimulated recycling to a small degree, and Rab11S25N also inhibited recycling, but to a lesser extent than H13p (Figure 30B).

The results from the two sets of experiments, taken together, suggest that RCP plays a role in regulating the 'slow' recycling pathway i.e. transport that passes through the recycling compartment. This agrees well with the observation that RCP is a recycling compartment protein. The stronger inhibition observed with H13p, compared to the dominant negative Rab11 or Rab4 mutants, may be the result of a cumulative inhibition due to the sequestration of both Rab4 and Rab11 in an inactive complex. It is also possible that RCP binds to the other members of the Rab11 family. KIAA0941, KIAA0665, and Rip11 all interact with Rab25, in addition to Rab11 (Hales *et al.,* 2001; Prekeris *et al.,* 2001). Similar experiments that followed the recycling and degradation of ^{125}I-EGF, revealed that H13p inhibits the trafficking of the fraction of EGF that follows the recycling pathway, without affecting the degradation of the pool of EGF that follows the late endocytic pathway (data not shown).

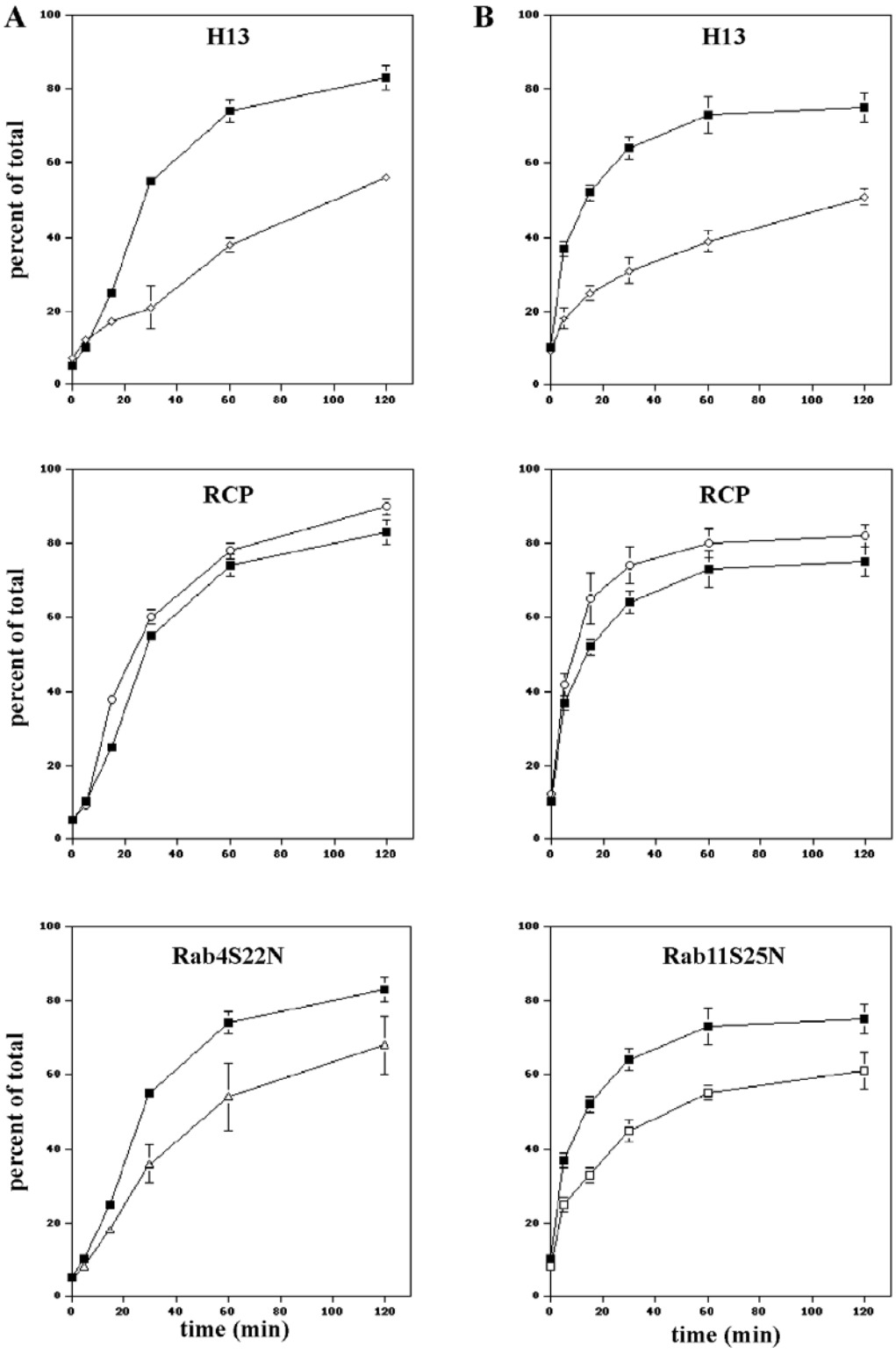


Figure 30. Kinetic analysis of the recycling of Tfn in cells overexpressing H13, full-length RCP, or dominant-negative Rab4 or Rab11. BHK cells were transfected with the human TfnR alone (control, ■) or together with H13 (◊), full-length RCP (○), Rab4S22N (Δ), or Rab11S25N (□). **(A)** Cells were allowed to bind ^{125}I-Tfn for 1 hour on ice. Excess ligand was removed and cells were incubated at 37^{o}C for the indicated times. **(B)** Recycling from the recycling compartment. Transfected BHK cells were

pulsed with ^{125}I-Tfn for 5 minutes. Excess Tfn was removed, and the cells were incubated at 37^{o}C for a further 30 minutes to chase the Tfn into the recycling compartment. Finally, the cells were washed again and incubated at 37^{o}C for the indicated periods of time. **(A** and **B)** At each time-point the amount of ^{125}I-Tfn in the culture medium was measured, and expressed as a percentage of the total ^{125}I-Tfn (i.e. total of cell associated ^{125}I-Tfn and ^{125}I-Tfn in the culture medium). In each panel the indicated construct is compared to the control results. The experiments were repeated 4 times and the bar is the SEM.

3.2 KIAA0941

3.2.1 KIAA0941 Sequence Analysis

KIAA0941 is a 511 amino acid protein, with a predicted molecular weight of 58kDa. It was deemed to be interesting since it shares a high degree of sequence homology with RCP (see Figure 19). It has an amino-terminal C2-domain (residues 17-103), and a carboxy-terminal putative coiled-coil domain;

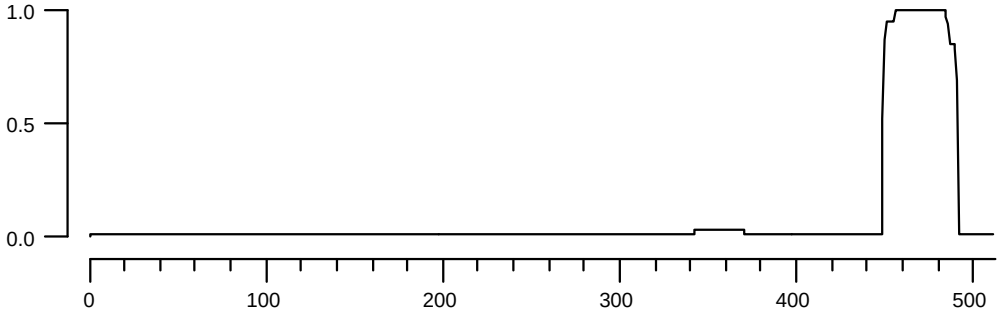


Figure 31. KIAA0941 has a putative coiled-coil domain at its carboxy-terminus. The *x*-axis indicates the residue number, and the *y*-axis indicates the probability of forming a coiled-coil (0-1).

This coiled-coil domain shares approximately 53% identity and 20% similarity with the Rab binding domain of RCP, suggesting that KIAA0941 may also interact with Rab4 and Rab11;

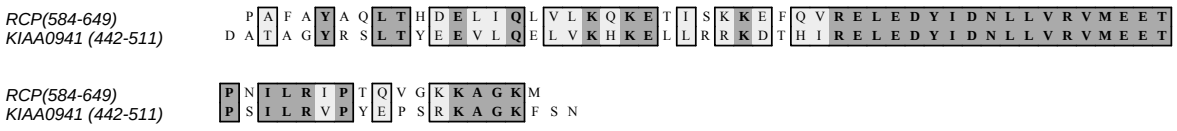


Figure 32. ClustalW alignment of the carboxy-termini of KIAA0941 and RCP. Identities are highlighted in dark grey, and similarities in light grey.

The KIAA0941 cDNA was requested from the Kazusa DNA Research Institute, Japan. The open reading frame was amplified and subcloned into the yeast two-hybrid 'prey' vector, pGADGH.

3.2.2 Yeast Two-Hybrid Analysis of KIAA0941/Rab Interactions

The KIAA0941 prey vector was co-transformed into *S.cerevisiae* L40 with Rab4WT and its mutants, or Rab11WT and mutants. An interaction between KIAA0941 and any of the Rab mutants was determined by spotting transformed colonies onto media lacking histidine. Surprisingly, considering the degree of homology with RCP, there was no interaction with Rab4. However, there was a strong nucleotide-independent interaction with Rab11;

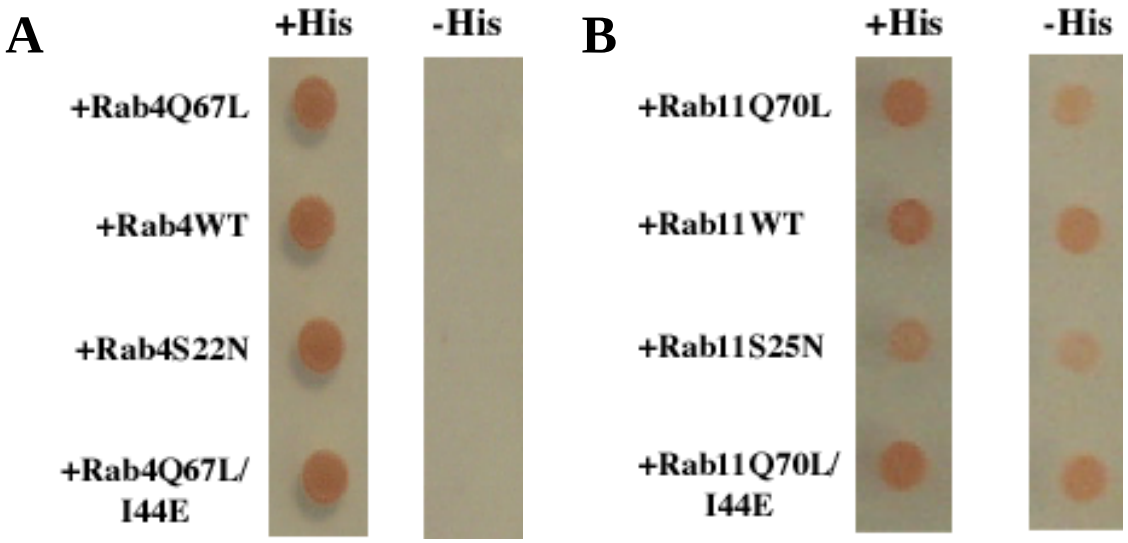


Figure 33. Analysis of the interaction between KIAA0941 and Rab4, or its mutants **(A)**, and Rab11, or its mutants **(B)**. An interaction is indicated by growth on media lacking histidine.

Quantitative β-galactosidase interaction assays revealed that KIAA0941 showed no significant preference for the nucleotide-bound state of Rab11. The strength of the interaction with all the Rab11 mutants was considerably higher than the RCP/Rab11 interaction (data not shown). There was no interaction between KIAA0941 and any of the other Rab GTPases tested (Rabs 3,5,6, and 7), although,

this does not rule out the possibility that it may interact with other recycling compartment Rab proteins, e.g. Rab15 (not tested) (Zuk and Elferink, 1999), or Rab proteins localised at the plasma membrane that function to accept transport vesicles from the recycling compartment.

Rip11, which also has a carboxy-terminal domain with high homology to RCP, also does not interact with Rab4 (unpublished results from Cell & Molecular Biology Lab., UCC). It is possible that Rip11 and KIAA0941 function after the Rab4-regulated delivery of material to the pericentriolar recycling compartment. They may function in the next step, the Rab11-regulated transport of material through the recycling compartment, or transport from the recycling compartment back to the plasma membrane, to the TGN, or elsewhere.

3.2.2.1 The Carboxy-terminal 66 amino-acids of KIAA0941 Mediate its Interaction with Rab11

To confirm that the carboxy-terminal coiled-coil domain of KIAA0941 mediates the interaction with Rab11, two truncation mutants were made in the KIAA0941 prey vector. These mutants took advantage of restriction enzyme sites within the KIAA0941 open reading frame. The amino-terminal mutant carried the first 445 amino acids of KIAA0941, but lacked the coiled-coil domain. The carboxy-terminal truncation expressed the final 66 residues that encompassed the coiled-coil domain. The ability of these two mutants to activate the transcription of the HIS3 gene was determined. From Figure 34 it can be seen that the carboxy-terminal region is sufficient to mediate the Rab11 interaction. This is an agreement with the results observed for the RCP interaction.

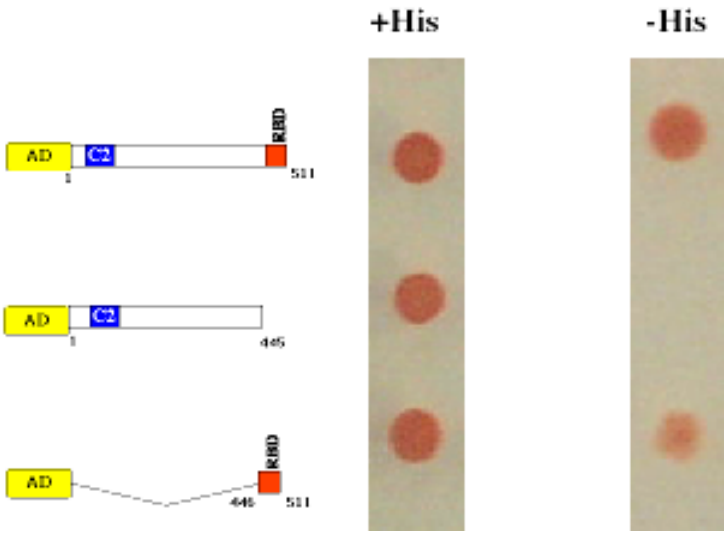


Figure 34. The carboxy-terminus of KIAA0941 mediates the interaction with Rab11. Full-length KIAA0941, or its truncation mutants, were co-transformed into *S.cerevisiae* L40 with Rab11WT. Interactions were detected by growth on media lacking histidine. Schematics represent the constructs tested. AD (Gal4 activation domain), RBD (Rab binding domain).

3.2.3 Expression and Purification of KIAA0941 Protein

The full-length KIAA0941 open reading frame was subcloned into pTrcHisC and expressed and purified from *E.coli* BL21 lysates;

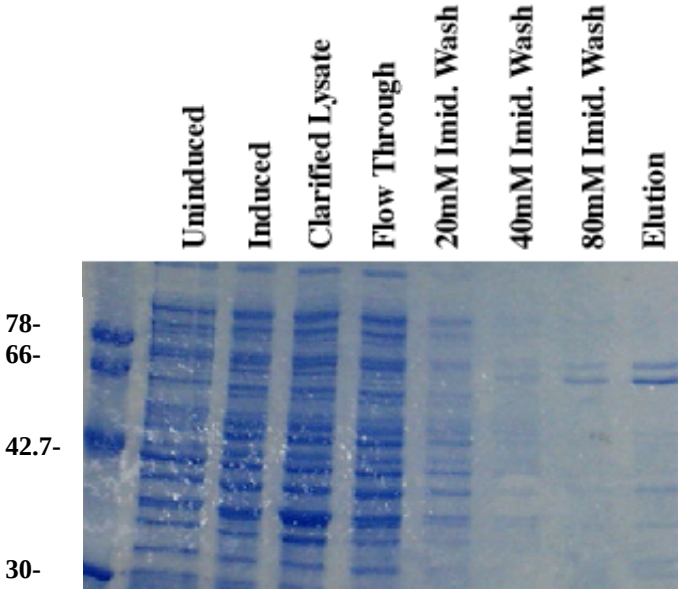


Figure 35. Coomassie Blue gel showing the steps involved in purifying 6XHis-KIAA0941FL.

To reduce the likelihood that antibodies raised against KIAA0941 may detect RCP it was decided to use the less homologous, amino-terminal region to inoculate rabbits. Using a suitable restriction enzyme site (HindIII) the carboxy-terminal 235 amino-acids were digested out of the expression vector and the backbone was religated. Following the purification procedure a doublet running at approximately 40kDa was eluted from the Ni^{2+}-agarose;

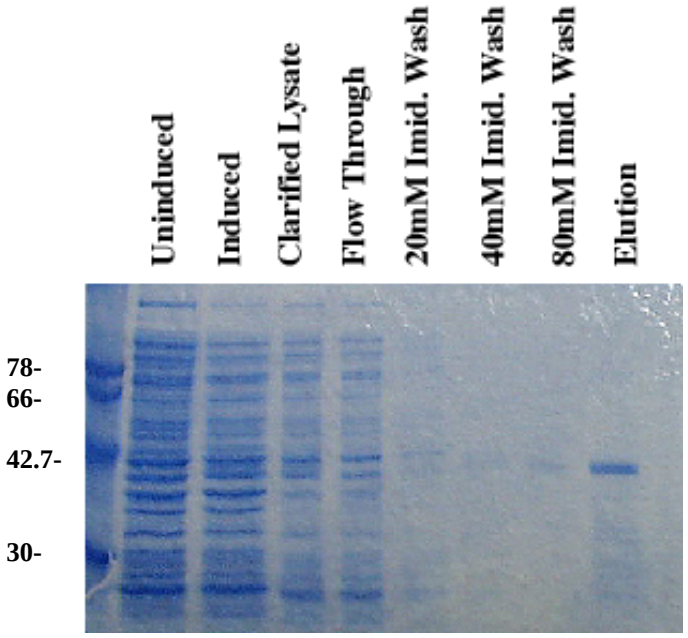


Figure 36. Coomassie Blue gel showing the steps involved in purifying 6XHis-KIAA0941NTp.

Immunoblotting with an anti-6XHis antibody revealed that both bands in the doublet carried a his-tag and both bands were expressed after induction with IPTG. Therefore, it is likely that the lower band is a degradation product of KIAA0941NTp. This preparation of KIAA0941NTp was used to inoculate a New Zealand White rabbit. The rabbit was given a series of 6 injections of the recombinant protein (250μg per injection) over eight weeks.

The carboxy-terminal 235 amino acids of KIAA0941 (KIAA0941CTp) were also purified;

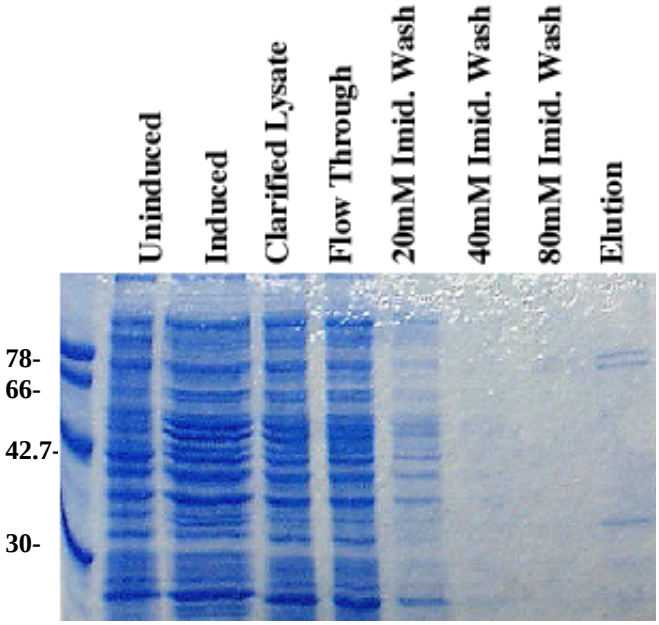


Figure 37. Coomassie Blue gel showing the steps involved in purifying 6XHis-KIAA0941CTp.

The resulting polypeptide was approximately 33kDa in size. The majority of KIAA0941CTp was insoluble, however, there was a significant proportion in the soluble fraction, which allowed low amounts of the truncation mutant to be purified.

3.2.3.1 KIAA0941 Antiserum

A 1 in 500 dilution of the antiserum raised against KIAA0941Ntp detected a strong approximately 66kDa band in 25μg of HeLa postnuclear supernatant (Figure 38). To determine whether the H13p serum cross-reacted with KIAA0941 and vice versa, increasing amounts of a bacterial lysate expressing full-length His-KIAA0941, or GST-δRCP, were transferred to nitrocellulose and probed with the opposing antiserum. Neither antiserum showed any cross-reactivity (Figure 39).

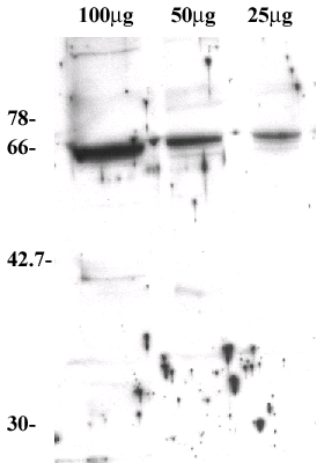


Figure 38. KIAA0941 antiserum detects a 66kDa band in HeLa PNS. The amount of HeLa PNS loaded is indicated above each lane.

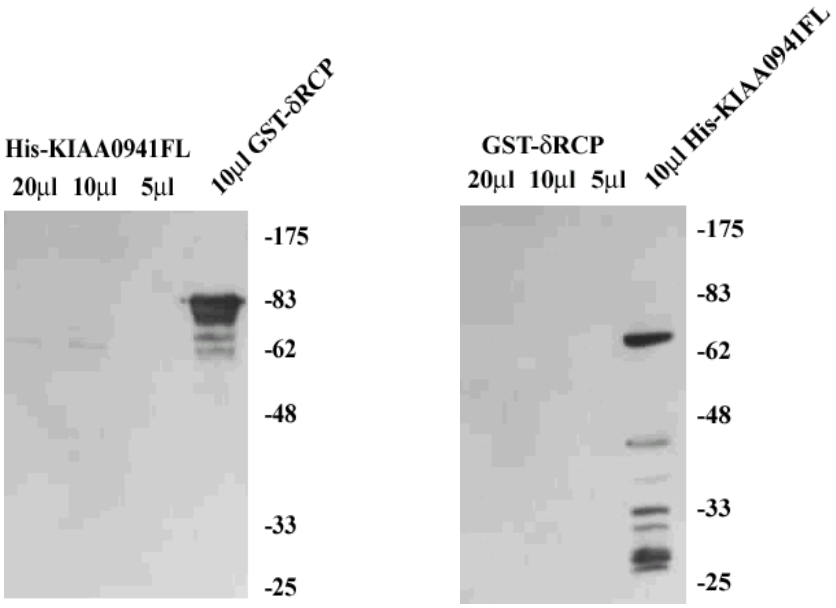


Figure 39. H13p and KIAA0941Ntp antisera do not cross-react. **(A)** H13p antiserum (1:1000) does not detect His-KIAA0941FLp. GST-δRCP was used as a positive control. **(B)** KIAA0941p antiserum (1:500) does not detect GST-δRCP. His-KIAA0941FLp was used as a positive control. The amounts of *E.coli* lysate in each lane are indicated above.

3.2.4 KIAA0941 interacts with Rab11 in vitro

To determine if KIAA0941 interacts with Rab11 *in vitro*, and to determine if the interaction is a direct protein-protein interaction, a Far Western technique was employed. Approximately 2µg of purified full-length KIAA0941p, KIAA0941NTp, and KIAA0941CTp were run on an SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose was incubated overnight in 'Renaturation Buffer'. 10µg of purified GST-tagged Rab11Q70L was incubated with cold GTP. The GST-Rab11Q70L was used to probe the nitrocellulose. Any Rab11 that was bound to the immobilised KIAA0941 recombinant proteins, after several wash steps, was detected with an anti-GST antibody (Amersham Pharmacia) (Figure 40). Rab11 bound to full-length KIAA0941p and the carboxy-terminal fusion protein, but did not bind to the amino-terminus of KIAA0941. This confirms the yeast two-hybrid results.

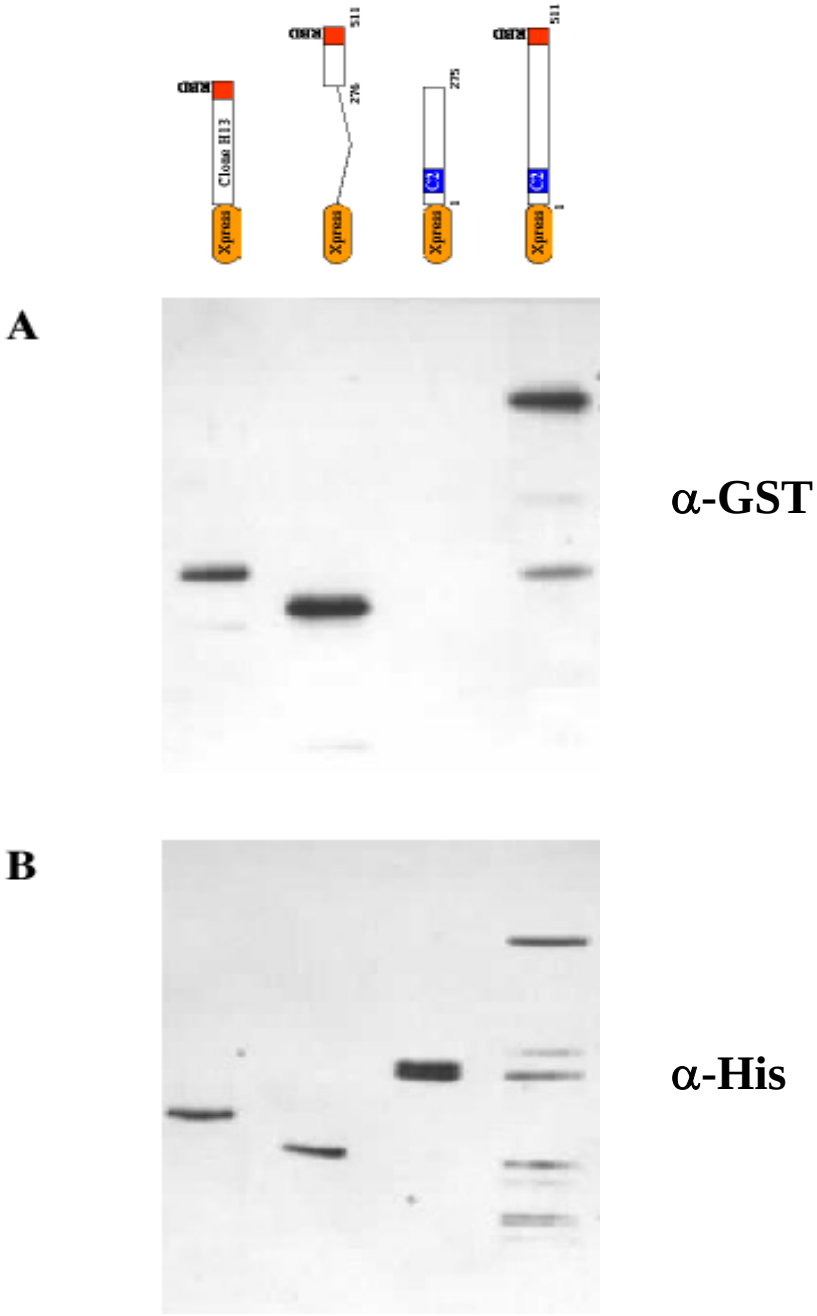


Figure 40. Rab11 interacts with KIAA0941 *in vitro.* **(A)** Far Western blot; bound Rab11 was detected with an α-GST antibody. **(B)** Western blot; an α-His antibody

was used to show loading of full-length KIAA0941p and its truncation mutants. H13p is loaded as a positive control. Schematic indicates the construct loaded in each lane.

3.2.5 Green Fluorescent Protein-tagged KIAA0941 Localises to Vesicular Structures in the Perinuclear Region of HeLa Cells

To determine the localisation of KIAA0941 within the cell, the full-length protein was subcloned into pEGFP-C1 (Clontech). This vector adds an enhanced green fluorescent protein (EGFP) fusion to the amino terminus of KIAA0941. The GFP tag enables the direct visualisation of KIAA0941 in HeLa cells without the necessity for antibody staining procedures. The construct was transfected into HeLa cells using Effectene (Qiagen), and after 6 hours it was possible to observe the GFP-KIAA0941 localising to distinct vesicles around the perinuclear area of the cell (Figure 41A). There appeared to be very few labelled vesicular structures in the periphery of the cells.

The observation that KIAA0941 interacts with Rab11, and by analogy with its homologue RCP it would be likely that the perinuclear vesicles labelled by the GFP construct are part of the recycling compartment. To confirm this, cells expressing GFP-KIAA0941 were labelled for the TfnR and Rab11. There was partial colocalisation with the TfnR (Figure 42A), and almost complete colocalisation with Rab11 (Figure 41A). In the Rab11 experiment, similar to the effect seen upon expression of RCP, Rab11 appeared to be recruited to the GFP-KIAA0941 positive vesicles.

3.2.6 Localisation of the Amino-terminal and Carboxy-terminal Regions of KIAA0941

To investigate the roles of the amino- and carboxy- terminal domains of KIAA0941, truncation mutants were constructed that expressed, either the carboxy-terminus, pEGFP-KIAA0941Ct (amino acids 446-511), or the amino-terminus, pEGFP-KIAA0941Nt (amino acids 3-445), of KIAA0941 fused to GFP. The GFP-

KIAA0941Nt construct contained the C2-domain, leucine-zipper, and a large part of the nonhomologous region that separates the two domains. The GFP-KIAA0941Ct mutant contained the coiled-coil Rab binding domain. Expression of the carboxy-terminus mutant in HeLa cells resulted in a similar pattern to the full-length protein. The construct labelled perinuclear vesicular structures. Interestingly, in some cells these structures appeared to be in the process of elongating (Figures 41C & 42C). This phenotype is probably the early stages of the similar phenotype seen upon H13p overexpression. This result indicates that the carboxy-terminal 66 amino acids of KIAA0941 are sufficient for the membrane localisation of KIAA0941, and also for the recruitment of Rab11.

It was considered likely that the C2-phospholipid binding domain of KIAA0941 may also have the ability to bind to membranes. However, expression of GFP-KIAA0941Nt resulted in a diffuse cytosolic pattern (Figures 41B & 42B). Since the strong cytosolic signal may have masked specific intracellular localisation of the truncation protein, cells transfected with GFP-KIAA0941Nt were permeablised prior to fixation. This washes out cytosolic protein and allows visualisation of protein that is anchored to a specific lipid structure. However, prepermeablised cells showed no KIAA0941Nt fluorescence (data not shown). To overcome any hindrance that the relatively large GFP tag (approximately 23kDa) may have over the normal function of the C2-domain, the KIAA0941Nt construct was subcloned into the pcDNA3.1HisB vector. This vector adds a much smaller (approximately 3kDa) epitope to the amino-terminus of the construct. However, similar results to the GFP-KIAA0941Nt construct were observed (data not shown). Although it cannot be ruled out that the presence of any epitope disrupts the C2-domain, it appears that this domain cannot, alone, bind to the recycling compartment *in vivo*.

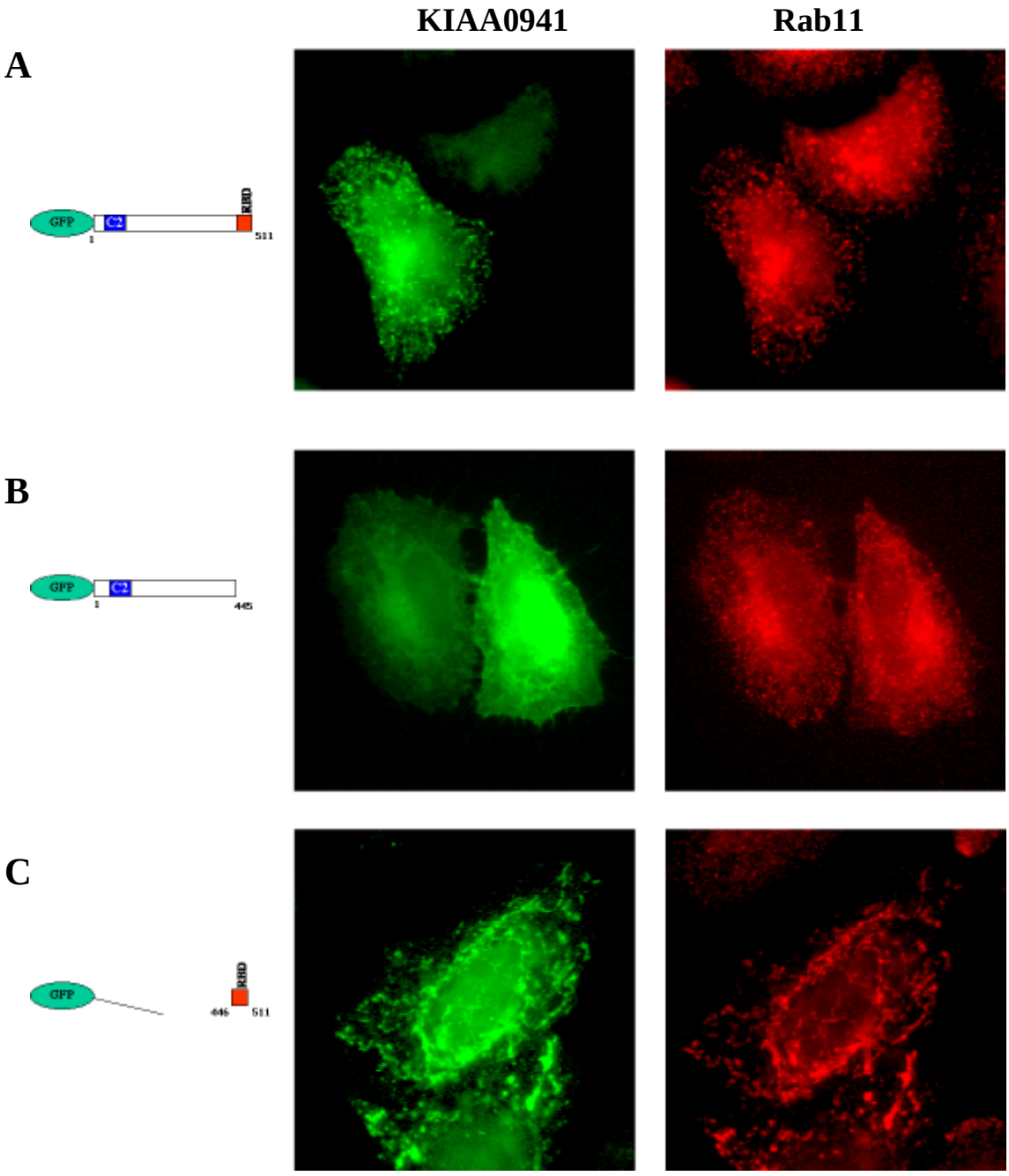


Figure 41. GFP-KIAA0941 and its truncation mutants localised with Rab11. HeLa cells were transfected for 6 hours with the indicated construct and labelled with anti-Rab11 (red). Schematics represent the constructs transfected.

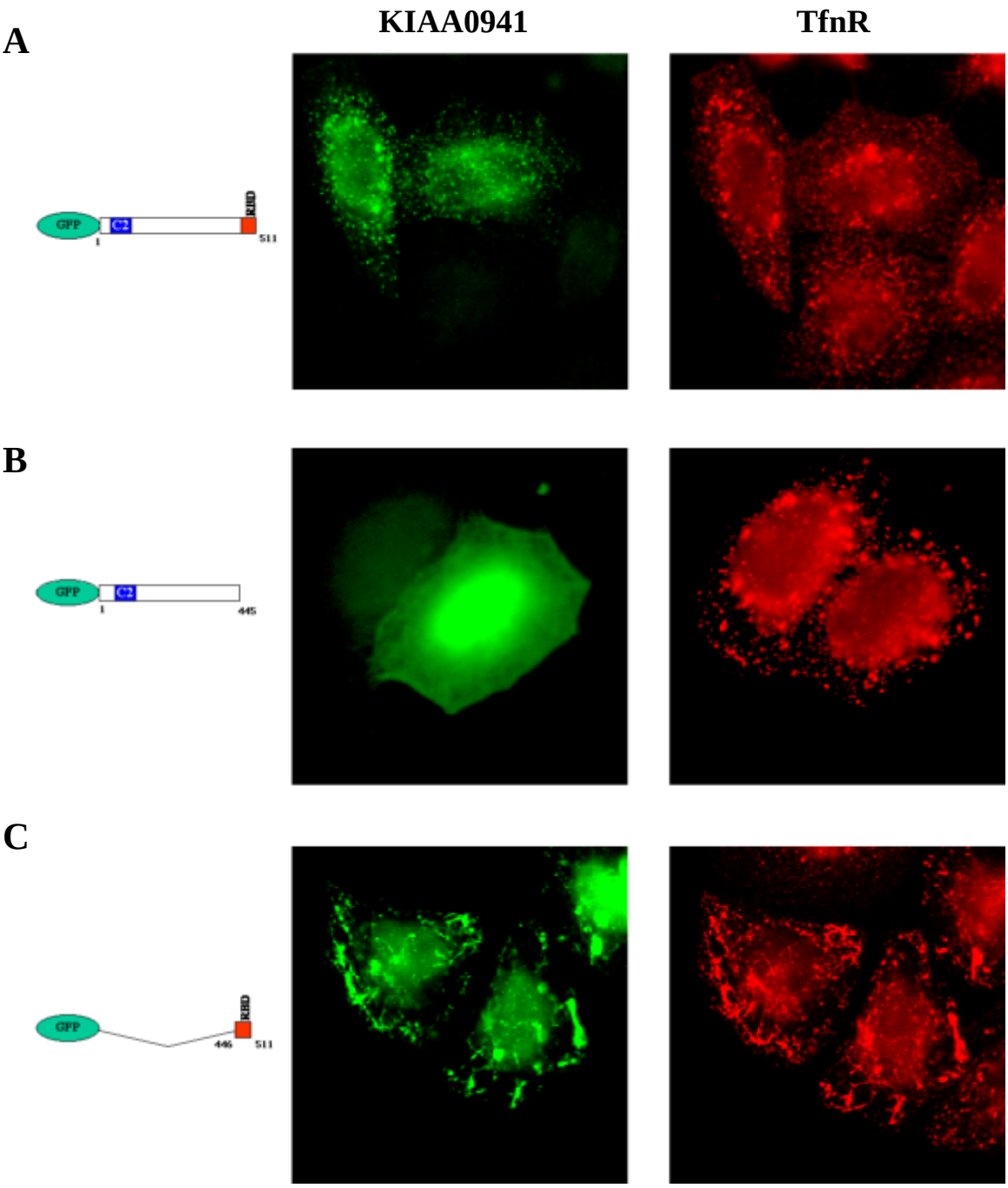


Figure 42. GFP-KIAA0941 and its truncation mutants localised with the TfnR. HeLa cells were transfected for 6 hours with the indicated construct and labelled with anti-TfnR (red). Schematics represent the constructs transfected.

3.2.7 Internalisation of FITC-Tfn in KIAA0941 Expressing Cells

To determine if the overexpression of KIAA0941 results in altered internalisation and recycling of FITC-Tfn in HeLa cells, the KIAA0941 open reading frame and its truncations were subcloned into the pcDNA3.1His vectors. The vaccinia system was used. In brief, HeLa cells were infected with the modified vaccinia virus, and transfected with the KIAA0941 constructs using the DOTAP transfection reagent. Ligand internalisation was performed six hours posttransfection.

3.2.7.1 Continuous Internalisation of FITC-Tfn at 37^{o}C

In cells expressing the KIAA0941Ct construct, FITC-Tfn accumulated in an extensive tubular network (Figure 43B), similar to that seen upon H13p-overexpression. This result was expected, and the tubules are likely to have been induced for similar reasons as the H13p-induced tubules i.e. it is possible that the KIAA0941Ct construct is sequestering endogenous Rab11 in an inactive complex, resulting in the inhibition of transport out of the recycling compartment. With transport into the recycling compartment occurring as normal, the compartment enlarges resulting in the formation of tubular structures.

In cells that overexpressed the full-length KIAA0941 construct there was a dramatic reduction in the amount of FITC-Tfn within the cell. To determine if this observation is exclusive to Tfn, co-uptake experiments with TxR-EGF and FITC-Tfn were performed in KIAA0941FL overexpressing cells. In these cells there was also a reduced level of TxR-EGF, when compared with non-transfected cells (data not shown). This phenotype is difficult to reconcile with the known functions of Rab11 and the localisation of KIAA0941. It is unlikely that KIAA0941 is inhibiting the internalisation of ligands from the plasma membrane since the protein appears to be localised to the recycling compartment, and Rab11 has not been implicated in the regulation of the early stages of endocytosis. The experimental design, i.e. continuous uptake of ligand, rules out the possibility that full-length KIAA0941 is stimulating recycling to such an extent that all of the ligand has been transported through the recycling pathway, and out of the cell, before the cells were fixed. One possible explanation is that overexpression of KIAA0941FL is toxic to the cell and that

endocytosis is shut down in these cells. In fact, when KIAA0941FL, or KIAA0941Nt, are transiently transfected and expressed from the CMV promoter for more than nine hours, cells start to die and after twenty-four hours the majority of the cells in the tissue culture plate have died.

3.2.7.2 Active Rab11 Does Not Rescue the KIAA0941Ct Phenotype

To determine whether KIAA0941 functions upstream or downstream of Rab11, the ability of Rab11 to reverse the phenotype induced by the dominant-negative KIAA0941Ct was tested. HeLa cells were co-transfected with KIAA0941Ct and Rab11WT (Figure 43C), or Rab11Q70L (Figure 43D), or co-transfected with KIAA0941Ct and the pGEM1 empty vector (Figure 43B). Six hours post-transfection the cells were allowed to internalise FITC-Tfn for 45 minutes at 37^{o}C. FITC-Tfn uptake in HeLa cells co-transfected with the parent vectors of the Rab11 (pGEM1) and KIAA0941 (pcDNA3.1HisB) constructs resulted in a pattern similar to that seen in non-transfected cells (Figure 43A). In cells expressing both KIAA0941Ct and active Rab11, a tubular phenotype was observed. This is in contrast with the results observed for the H13p-induced phenotype. These results suggest that KIAA0941 functions downstream of Rab11 in the receptor recycling pathway.

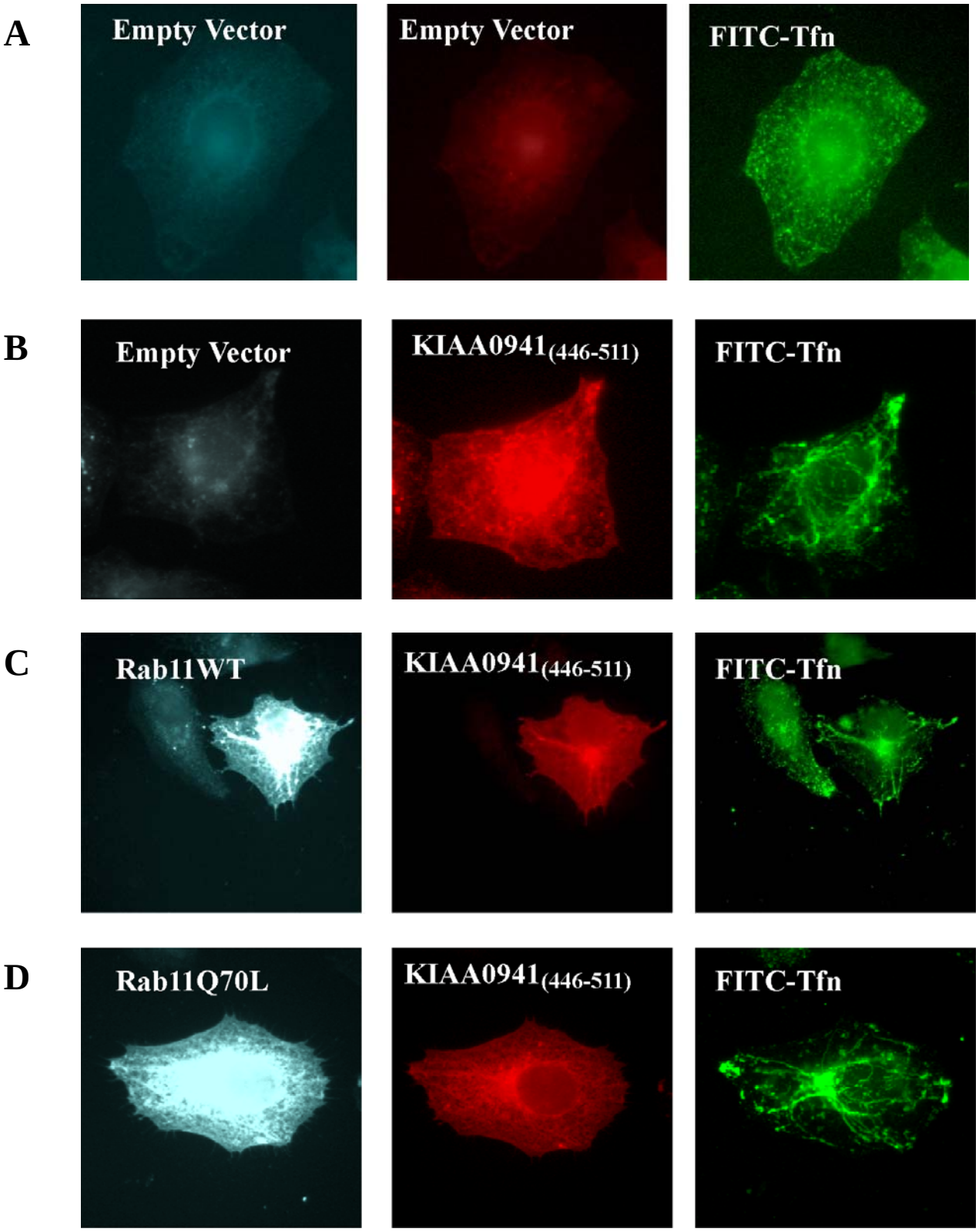


Figure 43. Active Rab11 does not rescue the KIAA0941Ct phenotype. FITC-Tfn (green) was internalised into cells co-transfected with pGEM1 and pcDNA3.1HisB **(A)**, pGEM1 and KIAA0941Ct (red) **(B)**, Rab11WT (cyan) and KIAA0941Ct (red) **(C)**, and Rab11Q70L (cyan) and KIAA0941Ct (red) **(D)**.

4 Discussion

4.1 The Identification of a Novel Rab4 Interacting Protein

The yeast two-hybrid technique is a powerful tool for identifying the binding partners of a protein of interest. It first proved invaluable for finding the downstream targets of small G proteins, when this method was used to identify Raf-1, a target for activated Ras (Vojtek *et al.,* 1993). Since then it has been used successfully to identify a number of Rab interacting proteins (Bielli *et al.,* 2001; Cormont *et al.,* 2001; Echard *et al.,* 1998; Janoueix-Lerosey *et al.,* 1995; Nagelkerken *et al.,* 2000; Stenmark *et al.,* 1995). It was decided to use the yeast two-hybrid technique to identify potential Rab4 effectors. The strategy adopted was to use a constitutively active mutant of Rab4, Rab4Q67L(-CXC), as the 'bait' to screen a HeLa cDNA library. Two groups of clones were identified in this screen. The first coded for a portion of the Rab5 effector, Rabaptin-5, a protein that had also been shown to interact with Rab4 (Vitale *et al.,* 1998). The second group, which included clone H13, contained identical inserts coding for the carboxy-terminal 270 amino acids of a novel protein. At the time of its isolation, this polypeptide had no homologues in the public databases. Analysis of the amino acid sequence identified a potential alpha-helical coiled-coil domain.

Further analysis of clone H13 in the two-hybrid system, demonstrated that the polypeptide behaved as a Rab4 effector i.e. it showed preferential binding to the GTP-bound form of Rab4. It did not interact with a mutant locked in the GDP-bound conformation, Rab4S22N, and the interaction was abolished by a mutation in the putative effector domain of Rab4. The clone was tested for its ability to interact with the constitutively active forms of other Rab GTPases. It displayed no interaction with Rab3, Rab5, Rab6, and Rab7. This result, and the nucleotide preference of the Rab4 interaction, suggested that clone H13 was a genuine Rab4 interacting protein and worth pursuing. An unexpected result was that the polypeptide displayed a strong interaction with Rab11, a recycling compartment Rab GTPase. This interaction was somewhat different than the interaction with Rab4, in that it did not appear to have a preference for the nucleotide-bound state of Rab11. To determine if the Rab11 interaction could be abolished by an effector domain mutation, a constitutively active mutant of Rab11, with a point mutation in its effector domain, was constructed. However, this mutation did not abolish the interaction, although, in quantitative assays the interaction strength was reduced by about one quarter. It has been

demonstrated that Rab4 and Rab11 show a significant overlap in their intracellular localisation (Sonnichsen *et al.,* 2000; Trischler *et al.,* 1999), and since they both have regulatory roles in the receptor recycling pathway, it was considered possible that these two Rab proteins share one or more binding partners. This had already been demonstrated for Rab5 and Rab4, both of which interact with Rabaptin-5.

4.2 RCP is a Recycling Compartment Protein

The clone H13 polypeptide (H13p) was expressed in a prokaryotic expression system and antibodies were raised against it. These antibodies were used to probe a number of cell lysates. A single band migrating at approximately 80kDa was detected in all the lysates tested. Since the H13 polypeptide was 37kDa, the two-hybrid clone did not code for the entire protein. The full-length protein was named Rab Coupling Protein (RCP), due to the observation that it interacts with two sequentially functioning Rab GTPases. After a number of unsuccessful attempts to isolate the full-length RCP cDNA, an expressed sequence tag (EST) that contained the full open reading frame, was deposited in the publicly available databases. The difficulty in cloning the 5'-end of RCP may have been due to a number of factors including the high GC-content at the 5'-end, and the presence of a NotI restriction site approximately 300bp from the initiating ATG. NotI is a restriction enzyme commonly used in the construction of cDNA libraries. The EST open reading frame was expressed in HeLa cells and the exogenous protein was shown to migrate at the same molecular weight as endogenous RCP.

Fractionation of HeLa cell lysates identified RCP as a predominantly membrane-bound protein, however, analysis of the amino acid sequence suggested that the protein is predominantly hydrophilic, with no potential transmembrane domains. Its membrane localisation is stable under high pH and salt conditions, but it is completely soluble in the presence of the detergent, Triton X-100, indicating that it is a peripheral membrane protein. It has a C2 phospholipid-binding motif at its amino-terminus, which may play a role in the subcellular targeting of RCP. The affinity-purified RCP antibody gave a vesicular pattern when used to detect the endogenous protein, by immunofluorescence, in HeLa cells. These punctate vesicles were seen throughout the cytoplasm, however they appeared to be more concentrated in the

perinuclear region. Colabelling experiments demonstrated that RCP partially colocalised with the TfnR and GFP-Rab4. To colocalise with Rab11, RCP was overexpressed at low levels and detected with a monoclonal antibody that recognised an amino-terminal epitope. Rab11 was detected with a rabbit polyclonal antibody. Under these conditions Rab11 displayed almost complete colocalisation with RCP. This result was all the more striking in that the cytosolic Rab11 appeared to be recruited by the recombinant RCP to the membranes. Rab11 displayed a much more diffuse pattern in cells that were not expressing the recombinant RCP. There appeared to be no similar recruitment of Rab4 from the cytosol.

To help decipher the function of RCP, the effect of the overexpression of H13p on the endocytic pathway was tested in HeLa cells. Fluorescently labelled Tfn accumulated in a complex tubular network in the cells that expressed the H13 polypeptide. The normal wild-type pattern was observed in non-expressing cells i.e. the Tfn displayed a much more vesicular pattern with a concentration of the ligand in the perinuclear region. EGF, the majority of which does not follow the recycling pathway did not accumulate in these tubular structures. Quantitative assays demonstrated that overexpression of H13p strongly inhibited the recycling of ligands. An interesting observation from the immunofluorescence experiments was seen in some cells that were minimally overexpressing H13p. The polypeptide in these cells was seen labelling the tubules. This indicates that the amino-terminal C2 domain is not a requirement for membrane binding. Thus, the carboxy-terminus, which contains the Rab binding domain, has the ability to bind membranes, either directly or via another membrane bound protein.

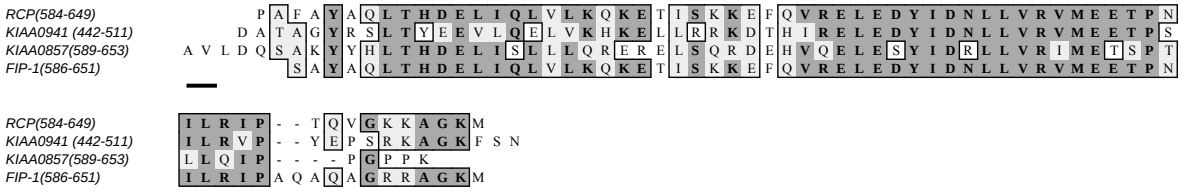
The almost complete colocalisation with Rab11, and the observation that there was very little overlap between RCP and EEA1, suggested that RCP is a recycling compartment protein. To test this further, the H13p-induced tubules were examined for their accessibility to internalised Tfn under different temperature conditions. When the fluorescent ligand was internalised at 18^{o}C it was unable to access the tubules, however, if the uptake at 18^{o}C was followed by a short chase at 37^{o}C, then the Tfn could be seen labelling the tubules. Transferrin is internalised into sorting endosomes at 18^{o}C, but transport out of the sorting endosome is blocked at this temperature (Wilcke *et al.,* 2000). From this result, it can be concluded that the H13p-induced tubules are derived from the recycling compartment. Co-expression of active Rab11 with H13p rescues the tubular phenotype.

4.3 RCP Belongs to a Family of Rab11 Interacting Proteins

Homology searches of the GenBank databases with the RCP amino acid sequence revealed that it has a number of human homologues. These include KIAA0857, KIAA0941, KIAA0665, KIAA1821, and Rab11-FIP1. KIAA0857 has since been reported as pp75 (Wang *et al.,* 1999), Rip11 (Prekeris *et al.,* 2000), and Gaf-1 (Chen *et al.,* 2001). Wang *et al.* reported KIAA0857 as a protein involved in systemic and neonatal lupus erythematosus, Prekeris *et al.* identified it in a screen for Rab11 effectors, and Chen *et al.* discovered it in a yeast two-hybrid screen for γ-SNAP interacting proteins. KIAA0941 interacts with Rab11 in a nucleotide-independent manner (see Results) and has since been reported as Rab11-FIP2 (Hales *et al.,* 2001), and nRip11 (Prekeris *et al.,* 2001). KIAA0665 interacts with Rab11-GTP, and has been named Rab11-FIP3 (Hales *et al.,* 2001), and Eferin (Prekeris *et al.,* 2001). Rab11-FIP1 was identified as a Rab11 interacting protein from a yeast two-hybrid screen by Hales *et al.*. KIAA1821 interacts preferentially with Rab11-GTP, and shows no interaction with Rab4 (personal communication, Debborah Wallace). Thus, RCP belongs to a family of Rab11 interacting proteins, but is unique in that it is the only member to also interact with Rab4 (it should be noted that Rab11-FIP1 has not been tested for an interaction with Rab4).

The members of this family are characterised by an α-helical coiled-coil domain, at their carboxy-termini, which mediates their interaction with Rab11. Prekeris *et al.* have mapped the Rab binding domain (RBD) to 20 amino acids;

A



B

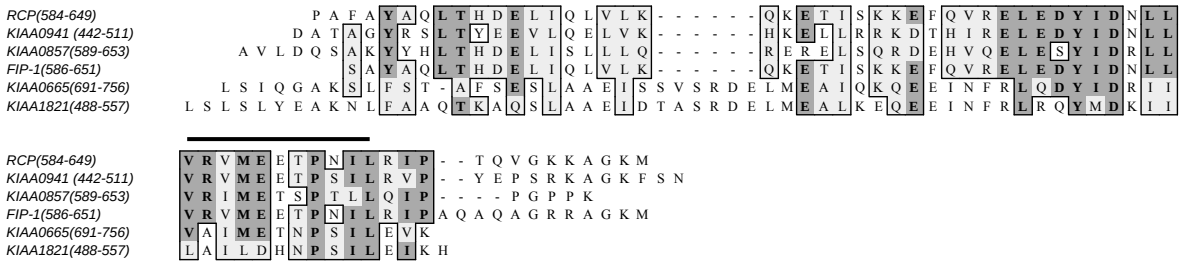


Figure 44. **(A)** ClustalW alignment of the coiled-coil domains of the close RCP homologues. **(B)** ClustalW alignment including the more divergent members of the RCP-family, KIAA0665 and KIAA1821. The line indicates the minimal RBD identified by Prekeris *et al.*.

Another characteristic of this family is that they possess (with the exception of Rab11-FIP1) a phospholipid-binding domain. RCP, KIAA0857, and KIAA0941, contain a C2 domain near their amino-termini. KIAA0665 and KIAA1821 carry EF-hand motifs (Figure 45). EF-hand motifs are generally short (approximately 30 residues) helix-loop-helix motifs that bind calcium or magnesium. They are the most common calcium-binding motif in mammalian cells. Interestingly, they have been found in myosin, and a pair of EF-hands make up the Eps15 homology (EH) domain (Lewit-Bentley and Rety, 2000). EH domains have been identified in the recycling compartment protein Rme1 (Lin *et al.,* 2001). KIAA0665 and KIAA1821 also possess ezrin/radixin/moesin (ERM) motifs just upstream of their RBD. ERM proteins are generally found in actin-rich cell surface structures where they function to cross-link actin filaments with the plasma membrane. They have been implicated in cell-cell adhesion, formation of microvilli, cell motility, and notably signal transduction pathways and membrane trafficking (Louvet-Vallee, 2000).

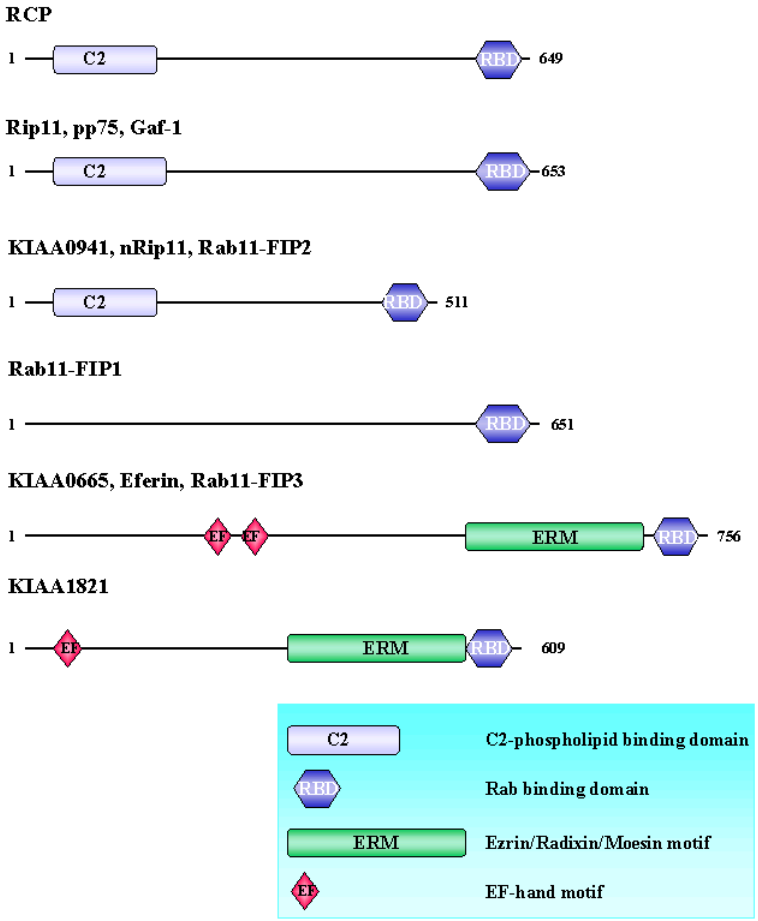


Figure 45. Schematic diagram depicting the domain organisation of RCP and its homologues. All names under which the individual proteins have been reported are indicated.

4.3.1 The Significance of the Phospholipid Binding Domains

It is becoming increasingly clear that a large number of effector proteins that are involved in endocytosis, possess phospholipid-binding domains. Such domains include FYVE (**F**ab1p, **Y**OTB, **V**ac1p, and **E**EA1; the first four proteins in which this domain was found), C2, pleckstrin homology (PH), PX (or Phox), and VHS (**V**ps27, **H**rs, and **S**TAM) domains. PH- and VHS- domains can bind PtdIns3*P*, but not with the same specificity as FYVE domains (see below). The PX-domain has recently been demonstrated to bind products of PI-3 kinase (Cheever *et al.,* 2001; Kanai *et al.,* 2001; Xu *et al.,* 2001). The approximately 130 residue conserved motif of the PX domain is found in proteins involved in cell signalling pathways, vesicular transport, and cell

polarity. There are at least 57 human, and 15 yeast proteins that possess PX motifs (Wishart *et al.,* 2001). C2 domains are approximately 130 residue motifs that can bind phospholipids, and in the majority of cases this lipid binding is Ca^{2+}-regulated. Multiple calcium ions can bind to C2 domains, which results in a structural rearrangement of the domain (Rizo and Sudhof, 1998). This is in contrast to the smaller EF-hand Ca^{2+} binding motif, which is generally a helix-turn-helix substructure in an α-helical domain and can only bind a single Ca^{2+} ion. C2 domains are found in a wide variety of proteins involved in cell signalling and membrane trafficking (Nalefski and Falke, 1996). Examples of C2 domain-containing proteins involved in membrane trafficking include the Rab3 effector, Rabphilin-3, which possesses two C2 domains (Shirataki *et al.,* 1993), and the synaptotagmins, which are transmembrane proteins localised to synaptic vesicles and are essential for the fast Ca^{2+} dependent neurotransmitter release (Sudhof and Rizo, 1996).

Since the identification of a FYVE domain on the Rab5 effector, EEA1, several other proteins involved in vesicular trafficking have been identified that contain these domains (Gillooly *et al.,* 2001). FYVE domains are cysteine-rich and can bind two zinc ions. Their function is to bind phosphotidylinositol 3-phosphate (PtdIns3*P*) (Gaullier *et al.,* 1998; Patki *et al.,* 1998). PtdIns3*P* is synthesised by phosphoinositide 3-kinases (PI 3-kinases) and is found largely in the sorting endosome membrane, although some PtdIns3*P* is found in the inner membranes of ECV/MVBs and lysosomes. Other sorting endosome FYVE domain-containing proteins include Rabenosyn-5 (Nielsen *et al.,* 2000), Rabip4 (Cormont *et al.,* 2001), and Hrs (Raiborg *et al.,* 2001b). The PI 3-kinase, hVPS34p, is recruited to the sorting endosome by Rab5-GTP, where it then synthesises PtdIns3*P,* thus facilitating the recruitment of FYVE proteins. While the FYVE domain specifically binds PtdIns3*P,* it is not sufficient on its own, for membrane recruitment. Rabip4 requires its FYVE domain and a RUN domain for correct localisation (Mari *et al.,* 2001), while Hrs requires the presence of the second of its two coiled-coil domains in addition to its FYVE-finger (Raiborg *et al.,* 2001b). EEA1 requires its Rab5 binding domain in conjunction with its FYVE finger. It can be hypothesised that the correct localisation of FYVE finger proteins is due to a combination of the FYVE domain and a specific protein-protein interaction. This may be the explanation why EEA1 is recruited specifically to the sorting endosome despite the fact that Rab5 is also found at the

plasma membrane and on CCVs (Rubino *et al.,* 2000). Hrs, while localising to the sorting endosome, is found on sub-domains distinct from those containing EEA1 (Raiborg *et al.,* 2001a). This is not surprising since Hrs does not interact with Rab5, but probably interacts with another sorting endosome protein via coiled-coil domain two. The reason why Rabip4 is not found associated with the recycling compartment pool of Rab4 may be due to its greater affinity for PtdIns3*P,* conferred by its FYVE domain.

The C2 domains of RCP, Rip11, and KIAA0941, may have a similar role in the specific targeting of these proteins to the recycling compartment. Prekeris *et al.* demonstrated that the C2 domain of Rip11 has an affinity for phosphotidylcholine (PC) and phosphotidylethanolamine (PE), but shows very little binding to PtdIns3*P* (Prekeris et al., 2000). They also demonstrated that Rip11 phospholipid binding required Mg^{2+} instead of Ca^{2+}. If the recycling compartment membrane possesses PC/PE-rich domains, this would provide a mechanism for the fine subcellular localisation of these proteins. The EF-hands of KIAA0665 and KIAA1821 may mediate the binding, directly or indirectly, to a different set of phospholipids found on a different sub-domain of the recycling compartment, or on a different compartment on which Rab11 resides e.g. the TGN or the plasma membrane. In this way, these Rab11 interacting proteins may maintain their specific localisation thus allowing for distinct roles, at distinct locations, in the endocytic pathway. To confirm this hypothesis it will be necessary to determine whether these proteins are mislocalised if either the phospholipid binding domain, or the protein interaction domain, is removed or mutated. The expression of truncation mutants of KIAA0941 in HeLa cells has indicated that its carboxy-terminal 65 amino acids, containing the Rab11 binding domain, are an absolute requirement for membrane binding. In fact, this small domain has the ability itself to bind membranes. Similar observations have been made for RCP, in that H13p can bind the endocytic membrane. However, it has yet to be determined whether the lack of a C2 domain results in the mislocalisation of these proteins on their target compartment.

4.4 The Biological Role of RCP and its Homologues

RCP is an 80kDa, predominantly membrane-bound, protein that interacts with both Rab4 and Rab11. The interaction with Rab4 is nucleotide-dependent, whereas RCP does not appear to have a preference for the nucleotide-bound state of Rab11. It is a recycling compartment protein, and overexpression of a truncated form of RCP, that contains the Rab binding domain, causes the recycling compartment to form an extensive tubular network. This tubular phenotype can be rescued by adding active Rab11 to the system. Quantitative transport assays have demonstrated that overexpression of H13p results in a strong inhibition in the recycling of ligands, without affecting their uptake or degradation. RCP has a phospholipid binding, C2-domain, near its amino-terminus.

How can these pieces of data fit together into a model to explain RCP function along the recycling pathway? The observation that RCP interacts with two Rab GTPases that function sequentially along the receptor recycling pathway suggests that it acts as a 'molecular bridge' between the transport step regulated by Rab4 and the step regulated by Rab11. RCP may be the 'tethering molecule' speculated by Zerial *et al.* to link the Rab4 and Rab11 membrane domains (Sonnichsen *et al.,* 2000; Zerial and McBride, 2001). The sequential action of two GTPases was first demonstrated by Wickner and colleagues, using a yeast homotypic vacuole fusion assay (Eitzen *et al.,* 2000). The yeast Rab homologue, Ypt7p, associates with effector proteins of the HOPS complex (Vps proteins 11, 16, 18, 33, 39, and 41) to reversibly tether vacuoles. Using Gdi1p, a GDI that can extract Ypt7p from membranes, the authors demonstrated that Ypt7p is necessary for the docking of vacuoles. However, once docking has occurred, the fusion reaction becomes insensitive to Gdi1p, but remains sensitive to GTPγS. These results suggest that Ypt7p is required for the docking step but not for the subsequent fusion reaction. Another GTPase is required for the fusion reaction since fusion is inhibited by the presence of nonhydrolysable analogues of GTP.

If Rab4 and Rab11 have an analogous, sequential, role in regulating the docking and fusion of transport vesicles with the recycling compartment, RCP may function as the link between these two small GTPases. This would be consistent with the data accumulated to date. RCP is localised to the recycling compartment, and interacts

preferentially with Rab4-GTP. Therefore, it is likely that RCP acts as a target for the pool of active Rab4 that is present on transport vesicles destined for the recycling compartment. RCP may then recruit Rab11 to the site of vesicle docking, where Rab11 becomes activated and takes over from Rab4, to regulate the next step of the process. This would explain the lack of nucleotide specificity of the RCP/Rab11 interaction, since it would be necessary for RCP to have the ability to bind to Rab11-GDP. RCP itself, or other Rab4 and Rab11 effector proteins, may interact with components of the SNARE machinery, thus linking the Rab and SNARE complexes.

This hypothesis would fit in with the model proposed by Marino Zerial that describes the coordination of Rab-dependent membrane tethering and docking, with SNARE-dependent fusion (Zerial and McBride, 2001). This model proposes that *cis*-SNARE complexes are selectively incorporated into Rab domains via interactions with Rab effectors. Once activated by the Rab effectors, the cognate SNAREs are able to form a *trans*-SNARE complex, which then drives membrane fusion. There are a number of layers of regulation that may occur. One, the Sec1p family of proteins can regulate SNARE pairing by sequestering syntaxin molecules. Rab effectors such as the HOPS complex and Rabenosyn-5 have been shown to interact with Sec1p-family proteins (Nielsen *et al.,* 2000). They may therefore have a role in removing the Sec1p block on SNARE pairing, or they may deliver Sec1p to *trans*-SNARE pairs, thus stabilising them (Figure 46A). Another mechanism of regulation may involve a series of interactions that have been observed between Rab effectors and the SNARE priming machinery. The assembly of the EEA1 and Rabaptin-5/Rabex-5 oligomers, on the sorting endosome, is regulated by the ATPase activity of NSF. NSF in complex with α-SNAP binds to docked SNARE complexes and promotes fusion (McBride *et al.,* 1999). The presence of NSF within the Rab effector oligomer complexes could function to site-specifically prime SNAREs, or to regulate the interactions between SNAREs and Rab effectors. Another SNARE priming protein, γ-SNAP, a relative of α-SNAP, has been shown to interact with Rip11 (Chen *et al.,* 2001). (Figure 46B). Finally, Rab effectors have been shown to interact directly with SNAREs. For instance, EEA1 binds to syntaxin 13 (McBride *et al.,* 1999), and the HOPS complex of Ypt7p has been found associated with the Vam3p SNARE complex (Figure 46C). Rab4 interacts directly with the plasma membrane t-SNARE, syntaxin 4 (Li *et al.,*

2001). It will be necessary to determine if RCP interacts directly, or indirectly, with components of the SNARE machinery, before it can be placed in this model.

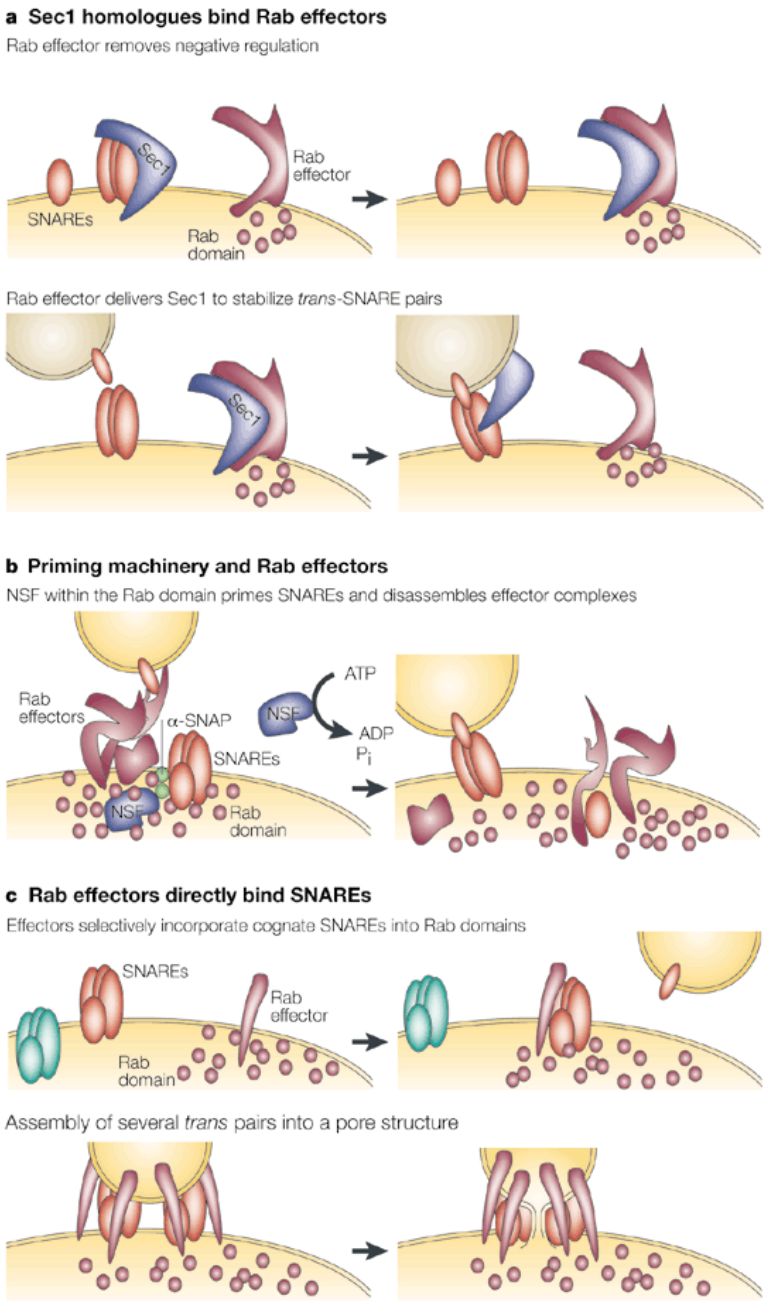


Figure 46. Molecular interactions showing coordination between the Rab and SNARE machineries (Zerial and McBride, 2001).

The above model for RCP function is speculative and, therefore, it cannot be ruled out that RCP plays no part in the docking/fusion process, or that it has other roles in conjunction with a role in docking/fusion. One such role could be in mediating vesicle motility. Rab4 interacts with the dynein light intermediate chain-1

subunit of the dynein motor protein, and is thought to mediate the retrograde cytoskeletal transport of Rab4-positive vesicles (Bielli *et al.,* 2001). The unconventional myosin, myosin Vb, has been found to interact with Rab11 (Lapierre *et al.,* 2001). The class V unconventional myosins have been implicated in the regulation of vesicle trafficking, and recent work has reported a stoichiometry of 100 myosin Va molecules per brain vesicle. Therefore, it has been proposed that myosin Vb, via its interaction with Rab11, is involved in exit from the recycling system. Recently it has been shown that myosin Vb also interacts with KIAA0941, thus there is a direct, and an indirect link, between this motor protein and Rab11 (Hales *et al.,* 2001). Rab4 interacts directly with the light-intermediate chain 1 subunit of the dynein complex, however RCP does not interact with the dynein LIC1, or a partial clone, in the yeast two-hybrid system (unpublished data). An interaction with mysoin Vb has not been tested.

RCP has a number of potential phosphorylation sites, which may provide a means for regulating its function. Prekeris *et al.* have shown that phosphorylation plays a role in regulating the subcellular localisation of Rip11 (Prekeris *et al.,* 2000). Kinase inhibitors stabilise Rip11 on the membrane, and result in the redistribution of a large proportion of Rip11 to the plasma membrane. Conversely, treatment with phosphatase inhibitors results in the solublisation of Rip11. The authors propose the following model for Rip11 function:- Rip11 is recruited to the recycling compartment by Rab11-GTP, after budding of Rab11/Rip11 vesicles, Rip11 mediates their transport to the plasma membrane where it binds an unknown receptor, thus facilitating the pairing of cognate SNAREs, and subsequent membrane fusion. Hydrolysis of Rab11-GTP to GDP, and phosphorylation of Rip11, results in the redistribution of Rip11 from the plasma membrane to the cytoplasm, where it is free to return to the recycling compartment, after dephosphorylation, and enter another round of membrane trafficking. Whether RCP undergoes phosphorylation, and if so, does this result in relocalisation, is currently under investigation. The identification of the kinase involved would indicate the type of regulation exerted on RCP i.e. whether it can be regulated by extracellular signals, and/or by the cell cycle.

What roles do the other members of the RCP family play in Rab11 regulated transport? In addition to the recycling compartment, Rab11 has also been observed on the TGN and post Golgi vesicles (Urbe *et al.,* 1993). It can be postulated that if Rab11 is involved in regulating transport to, or from, these compartments, as well as to the

plasma membrane, then distinct Rab11 effector proteins are likely to be required for each of these pathways. The RCP family members may function as these effectors. RCP is involved in the early sorting endosome to recycling compartment pathway. Prekeris *et al.* have proposed that Rip11 is involved in transport from the apical recycling compartment to the apical plasma membrane in polarised cells, and presumably from the recycling compartment to the plasma membrane in non-polarised cells. The tubular phenotype observed upon overexpression of the Rab binding domain of KIAA0941, cannot be rescued by active Rab11, suggesting that it functions in a transport step downstream of RCP. Therefore, KIAA0941 may function in regulating transport through the recycling compartment, after which Rip11 takes over to regulate transport out of this compartment to the plasma membrane. Since KIAA0665 and KIAA1821 have a more divergent domain structure they may function to regulate transport to a different compartment, such as the TGN.

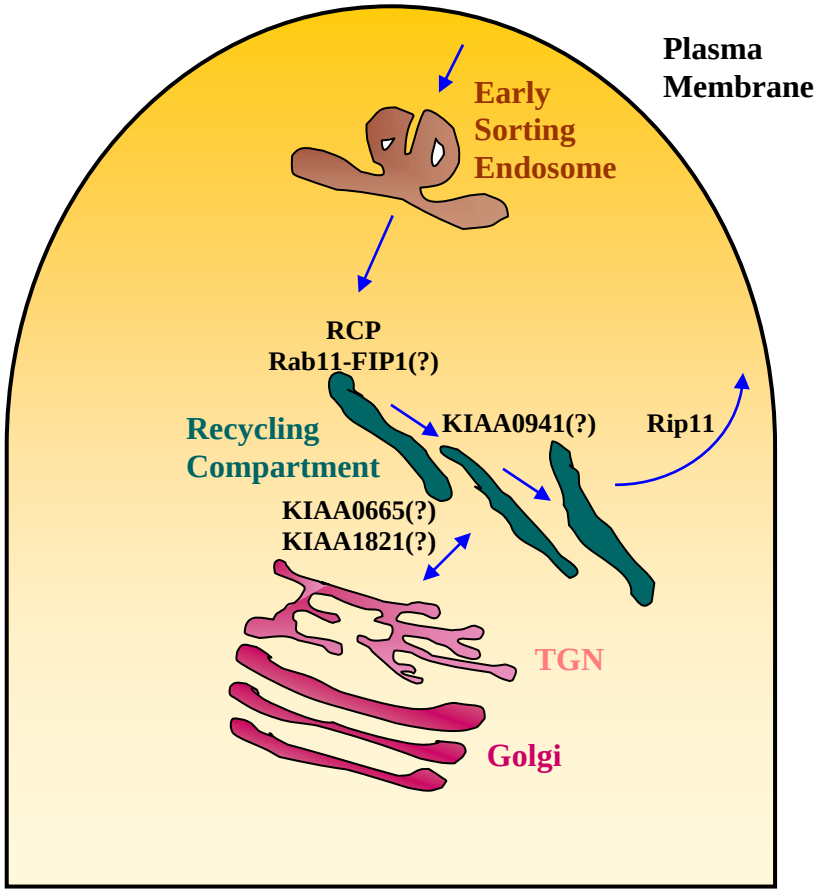


Figure 47. Schematic of the recycling pathway indicating the putative localisation of the RCP family members. Blue arrows indicate transport steps.

In a manner reminiscent of Rab5, it is emerging that both Rab4 and Rab11 interact with multiple effectors. It is likely that there are many more that remain to be reported. To obtain a more comprehensive understanding of the roles of Rab4 and Rab11 in endocytosis, it will be necessary to identify their complete repertoire of interacting proteins, and to determine the precise tasks that these effectors perform along the Rab4 and Rab11 pathways.

5 Conclusion

The yeast two-hybrid technique has allowed the identification of a novel Rab4 and Rab11 interacting protein. This 80kDa protein was named Rab Coupling Protein (RCP), due to the fact that it appears to couple the function of two sequentially acting Rab GTPases. It shows a preference for the GTP-bound form of Rab4, however, it interacts strongly with both GTP- and GDP-locked Rab11. RCP is a peripheral membrane-bound protein localised to the recycling compartment. Overexpression of a truncation mutant of RCP results in a dramatic alteration in the morphology of the recycling compartment and an inhibition in the rate of ligand recycling. The tubular phenotype induced by the truncation mutant can be rescued by active Rab11, but not by Rab4. It can be postulated that RCP acts as the recycling compartment acceptor for Rab4-GTP transport vesicles en route from the early sorting endosome, and that it recruits Rab11 (either from the cytosol, or the recycling compartment membrane) to the location where these transport vesicles dock/fuse with the recycling compartment. Rab11 is then in a position to take over the regulation of the transport of cargo contained within these transport vesicles.

Homology searches of the GenBank databases with the RCP amino acid sequence identified a number of close human homologues, which make up a protein family. The members of this RCP-family are characterised by an α-helical coiled-coil domain at their carboxy-terminus, and either a phospholipid binding C2-domain near their amino-termini, or a calcium-binding EF-hand motif. Analysis of these homologues has demonstrated that they all interact with Rab11, but RCP is unique in that it is the only member tested that also interacts with Rab4. These family members show no significant homology with either Rabphilin-11 or Myosin Vb, two other Rab11 interacting proteins. The RCP homologues are each likely to function in distinct transport steps regulated by Rab11.

Future work will be required to dissect the precise subcellular localisation of the RCP-family members to determine whether they localise to separate subdomains of the recycling compartment, or whether the more divergent members localise to a different compartment. It is likely that the C2- and EF-hand domains are involved in 'fine-tuning' this localisation, therefore it will be necessary to determine the specific phospholipids to which they bind. An important question remaining concerning RCP is whether Rab4 and Rab11 can bind simultaneously to the same molecule, or whether they share a binding motif on RCP, and therefore the presence of one GTPase would

exclude the binding of the other. To further characterise the RCP-family proteins it will be crucial to identify the proteins through which they mediate their downstream functions.

In conclusion, the identification of RCP and its homologues has added further data to the picture that is emerging concerning the biological roles of Rab4 and Rab11 along the recycling pathway, and as is the case with the rapidly progressing life sciences, it has opened up more avenues of research that need to be explored.

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