The *Dictyostelium discoideum* RACK1 orthologue has roles in growth and development

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Abbreviations

Dd	*Dictyostelium discoideum*
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
GpbB	G protein beta subunit B
GPCR	G-protein-coupled receptor
GST	Glutathion-S-Transferase
IPTG	Isopropyl-thio-galactoside
kDa	Kilo Dalton
min	Minute
mM	Millimolar
M	Molar
PAGE	Polyacrylamide Gel electrophoresis
PCR	Polymerase chain reaction
PIPs	Phosphoinositides
PMSF	Phenylmethylsulfonyl fluoride
RACK1	Receptor for activated C Kinase 1

RFP	Red fluorescent protein
RpkA	Receptor Phosphatidylinositol Kinase A
SDS	Sodium dodecyl sulphate
TAE	Tris-Acetate-EDTA-Buffer
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
v/v	volume per volume
YFP	Yellow fluorescent protein
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside

1 Introduction

1.1 The WD-repeat family of proteins

The tryptophan, aspartic acid-repeat (WD-repeat) containing proteins are an ancient conservative family of proteins found in prokaryotes and all eukaryotes (Li and Roberts, 2001). They are involved in almost every signalling pathway and are associated with many genetic diseases. Until now over 100 WD-repeat proteins have been assigned with an approved name and designation in the human nomenclature database (Adams *et al*., 2011). WD-repeats themselves are sequences of typically 40-60 amino acids in length ending at the C-terminus with a signature WD dipeptide or its equivalent (Adams *et al*., 2011). The motifs were first identified as repeating segments of homologous sequence within the primary structure of the transducin Gβ subunit and CDC4 (Fong *et al*., 1986). The WD-repeats are also characterised by the presence of a GH dipeptide which is usually in the N-terminus, though neither the GH nor the WD dipeptide is absolutely conserved. Moreover, WD-repeat proteins may contain N-terminal and C-terminal regions of variable length (Chen *et al*., 2004). Several other characteristic amino acids contribute to the repeat, most notably an aspartic acid located 6 residues before the WD dipeptide, but it is the collective critical mass of such features rather than the absolute conservation of any individual amino acid that establishes the identity of a sequence as a WD-repeat (Xu and Min, 2011). Given the variable number of residues at the N-terminal end of these units, sequence databases tend to map the repeats of WD-proteins between GH and WD dipeptides for convenience (Adams *et al*., 2011). The basic criterion for inclusion of a protein into this family is the presence of at least four of these repeat sequences to generate a WD-domain. These domains adopt a β-propeller structure, where the propeller fold is characterised by blades that are arranged radially around a central axis (Figure 1) (Neer *et al*., 1994; Neer, 1995; Paoli, 2001; Sondek *et al*., 1996; Wall *et al*., 1995).

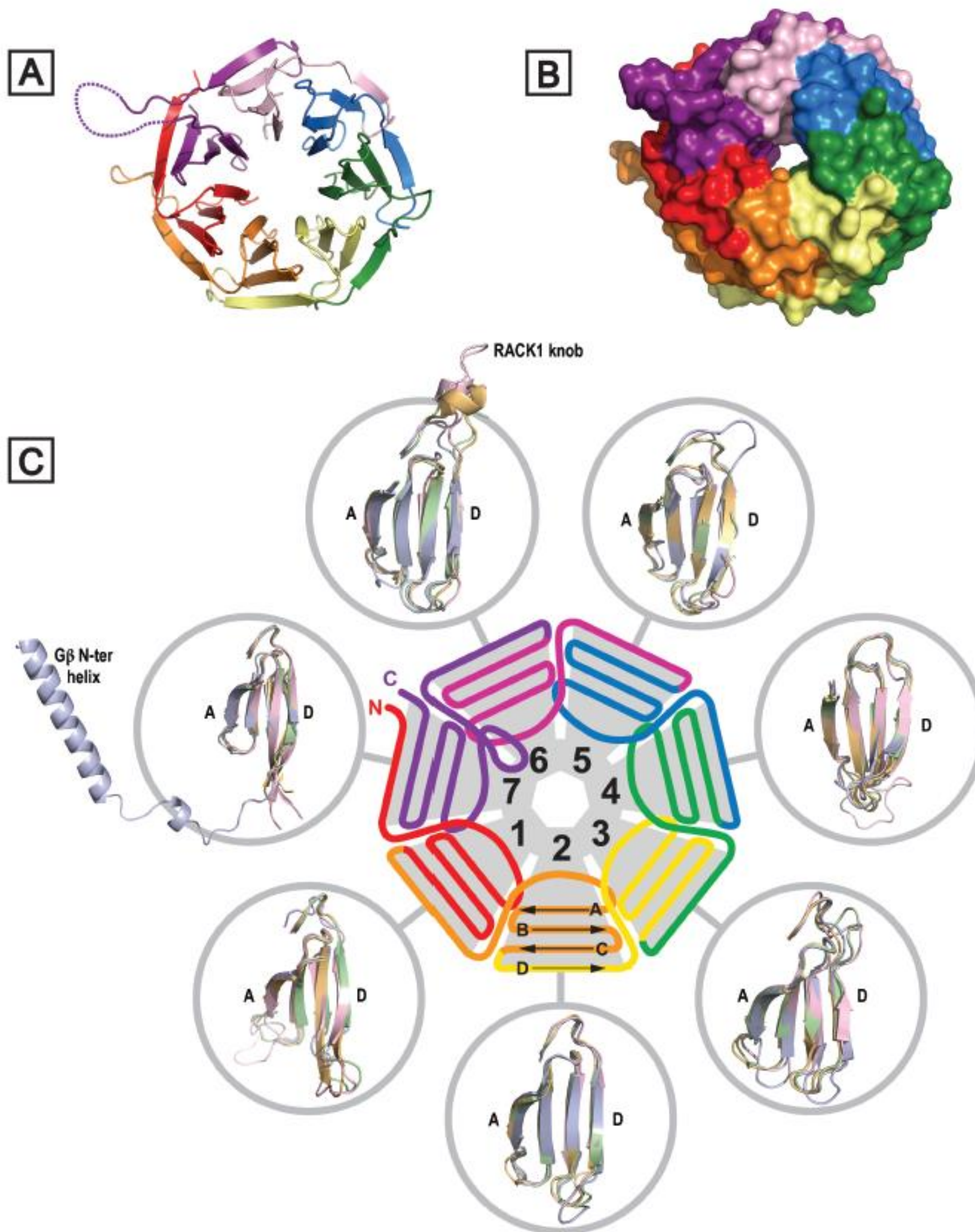


Figure 1 Structural detail for RACK1 proteins. (A) Crystal structure of RACK1A from *A. thaliana* (PDB: 3DM0), illustrating the seven-bladed β-propeller structure. (B) As (A) but with surface rendition. (C) Schematic representation of RACK1 structure and organisation of WD-repeats; peripheral circles show superimposition of individual propeller blades for Gβ1 (blue) (PDB: 1TBG) and for the structurally defined RACK1

orthologues. Gβ is distinguished in blade 7 by a helical N-terminal extension that engages tightly bound Gγ (not shown) in coiled coil interactions (Adams *et al*., 2011).

The conserved propeller structure is maintained by well-defined hydrogen bonding networks and intra-chain hydrophobic interactions, although different WD-repeat proteins appear to adopt distinct folding orders for their constituent propeller blades (Sondek *et al*., 1996; Garcia-Higuera *et al*., 1998). In principle, s single β-propeller subunit may comprise four to eight blades (Smith, 2008; Paoli, 2001), although at present only 7-bladed or 8-bladed WD-repeat propellers have been characterised by X-ray diffraction studies-the majority being 7-bladed structures consistent with the proposal that this is the optimal β-propeller fold (Murzin, 1992). Proteins are known with more than eight WD-repeats, but these assume tertiary structures with multiple propeller subunits (Adams *et al*., 2011).

WD-repeat proteins share a common role in scaffolding protein complexes, often with multiple and competing partners, thereby serving as hubs for spatiotemporal orchestration of signalling events across diverse pathways. Enzymatic activities have not been reported thus far for WD-repeat proteins, but their functions can be regulated by post-translational modifications (Adams *et al*., 2011). The lack of a direct catalytic enzymatic function for the protein family contrasts with other β-propeller-forming proteins, many of which exhibit enzymatic activity (Paoli, 2001).

To date the most extensively studied WD-repeat protein is the G-protein β subunit (Gβ) (Neer, 1995; Sondek and Siderovski, 2001), which exists in a complex with the γ subunit (Gγ). Gβγ reversibly complexes with the GDP-bound Gα subunit to form a Gαβγ heterotrimer that associates with G-protein-coupled receptors (GPCRs) for transduction of extracellular signals. Agonist-bound GPCRs act as guanine nucleotide exchange factors (GEFs), promoting the release of the Gα subunit from the Gβγ heterodimer, resulting in the activation of various

signalling cascades (Schwindinger and Robishaw, 2001; Tuteja, 2009). The mode of Gβ interaction with its binding partners has become increasingly clear with the emergence of crystal structures of Gβ in various complexes with Gα/Gγ, (Sondek *et al*., 1996; Wall *et al*., 1995; Lambright *et al*., 1996; Nishimura *et al*., 2010), phosducin (Gaudet *et al*., 1996; Gaudet *et al*., 1999; Loew *et al*., 1998), GPCR Receptor Kinase 2 (GRK2) (Ladowski *et al*., 2003; Tesma *et al*., 2010; Tesma *et al*., 2005), and Regulator of G-protein Signalling 9 (RGS9) (Cheever *et al*., 2008; Slep *et al*., 2001). Evidence is accumulating for the homodimeric/heterodimeric interactions of the WD-repeat containing proteins including the Receptor for activated C kinase 1 (RACK1) (Thornton *et al*., 2004; Liu *et al*., 2007; Chen *et al*., 2004; Chen *et al*., 2005). Dimerization events are frequently employed to transduce signals from the cell surface to the nucleus (Klemm *et al*., 1998). Moreover, some WD-repeat proteins contains some other domains in addition to the WD repeat sequences that increase the number of binding partners, scaffolding properties and overall function of the protein (Adams *et al*., 2011). For example, the β-transducing repeat-containing protein 1 (β-TrCP1) is a ubiquitin ligase with both WD-repeat and F-Box domains as well as a RING domain. β-TrCP1 is required for the degradation of regulatory proteins such as Snail and p53 (Vinas-Castells *et al*., 2010; Xia *et al*., 2009), and the WD-repeats increase its binding cohort and regulate its sub-cellular location, allowing the protein to also have a role in transcription and in regulating circadian rhythm (Seo *et al*., 2009; Kimbrel and Kung, 2009; Ohsaki *et al*., 2008).

As the knowledge of the WD-repeat family of proteins increases, the members are being regularly shown to be involved in most signalling pathways. It is therefore not surprising to note that WD-repeat proteins play critical roles in several human diseases. It is also interesting

to know that changes in WD-repeat homeostasis can have a dramatic effect on protein complex assembly and on key signalling pathways.

1.2 Receptor for activated C kinase 1 (RACK1) - a scaffold protein: structure and physiological functions

Scaffold proteins are uniquely poised to integrate signals from multiple pathways. Such proteins generate considerable functional diversity by mediating concomitant and/or promiscuous interactions with a vast array of protein partners (Ullah *et al*., 2008). RACK1 is a highly conserved intracellular adaptor protein with significant homology to Gβ (Adams *et al*., 2011). RACK1 was originally cloned from a chicken liver cDNA library and human B-lymphoblastoid cell line (B-LCL) (Guillemot *et al*., 1989). The protein was then cloned from a rat brain cDNA library which was screened for gene products that bind purified rat brain PKC in the presence of its activators (phosphatidyserine, diacylglycerol and calcium) (Ron *et al*., 1994). Given the association of RACK1 with the active conformation of PKCβII, the protein was named Receptor for Activated C Kinase 1 (RACK1) (Ron *et al*., 1994; 1995; Ron and Mochly-Rosen, 1995; Stebbins and Mochly-Rosen, 2001).

It is now very well established that RACK1 interacts with a large number of proteins either directly or as a part of a complex. As a scaffold protein, RACK1 integrates inputs from distinct signalling pathways and is crucial for fundamental cellular activities such as cell proliferation, transcription and protein synthesis, as well as various neuronal functions (Adams *et al*., 2011). RACK1's scaffolding properties are mediated by the presence of seven WD-repeats (McCahill *et al*., 2002; Sklan *et al*., 2006) that present multiple protein-binding sites and facilitate interaction with specialized protein docking modules, including SH2 domains (Src and Fyn) (Chang *et al*., 2002; Yaka *et al*., 2002), pleckstrin homology (PH)

domains (dynamin and p120GAP) (Koehler and Moran, 2001; Rodriguez *et al*., 1999) and C2 domains (Ron *et al*., 1994; Stebbins and Mochly-Rosen, 2001).

RACK1 functions as a homodimer enabling the expansion of its binding partners (Chen *et al*., 2005; Thornton *et al*., 2004; Yaka *et al*., 2002; Dell *et al*., 2002; Liu and Semenza, 2007). The formation of the RACK1 heterodimer has been shown to enable efficient cross-talk between signal transduction pathways mediated by GPCRs and by ligand-gated ion channels, specifically between the cAMP/PKA pathway and the N-methyl D-aspartate receptor (NMDAR) (Thornton *et al*., 2004). RACK1 binding partners have been identified at various cellular locations. One very important function of RACK1 is to shuttle some of its interaction partners to specific sites within the cell. Global control of gene transcription, translation, and ribosome assembly are also emerging as important cellular functions of RACK1 (Ceci *et al.,* 2003; Nilsson *et al.,* 2004). RACK1 orthologs have also been discovered in lower eukaryotes, such as *Chlamydomonas* and yeast (Schloss, 1990), and are highly conserved in plants and other organisms (van Nocker and Ludwig, 2003), which do not express canonical protein kinase C enzymes (Herold *et al.,* 2002).

1.2.1 Structure of RACK1

Studies probing the evolution of RACK1 suggest that the protein is strongly conserved through evolution (Wang *et al*., 2003). Sequence alignments of RACK1 species from diverse organisms reveal a significant sequence identity. Gβ was the first WD repeat protein to be characterised by X-ray crystallography. Numerous crystal structures have since emerged for WD-repeat proteins (Sondek *et al*., 1996; Wall *et al*., 1995; Lambright *et al*., 1996; Stirnimann *et al*., 2010). These structural studies confirmed that RACK1 adopts a seven-bladed β-propeller structure consistent with the predictions of earlier homology modelling studies based on Gβ and other WD-repeat proteins (McCahill *et al*., 2002; Stirnimann *et al*.,

2010; Coyle *et al*., 2009; Rabl *et al*., 2011; Ullah *et al*., 2008; Ruiz *et al*., 2012). The *A. thaliana* protein was the first of RACK1 orthologues to be structurally defined. In contrast to metazoans, where RACK1 is expressed as a single gene, *A. thaliana* possesses three genes. The structure of human RACK1 is shown in Figure 2.

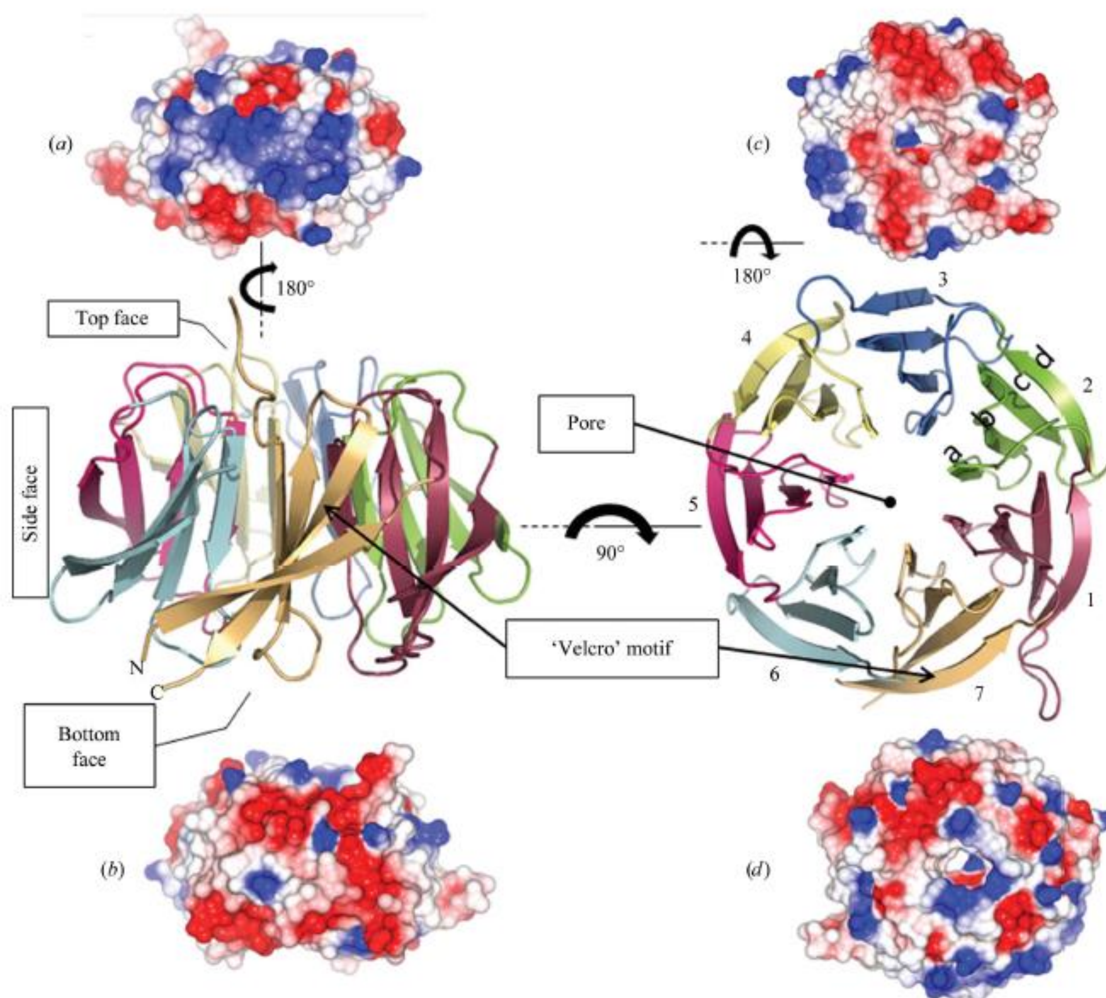


Figure 2 Views of the hRACK1 crystal structure. Central panel: cartoon representations of hRack1 viewed from the side (left) and from the top (right). The locations of the protein pore and the 'velcro' motif are indicated. Each β-sheet or blade is numbered sequentially from the N-terminus of the protein and their β-strands are labelled a, b, c,

and d starting from the inside of the propeller close to the pore. The electrostatic surface of hRack1 is shown from four different angles. Side surfaces, bottom and top views are displayed in (a) (blades 1–3), (b) (blades 5–7), (c) and (d), respectively (Ruiz *et al*., 2012).

Each β-sheet (1-7) shows the same basic architecture comprised of antiparallel strands labelled *a*, *b*, *c* and *d* from the inner side of the propeller to the outer side. Adjacent blades are connected by a loop bridging from strand *d* on one blade to strand *a* on the next. These loops are exposed on the top face of the propeller blade, as are the β-turns linking strands *b* and *c* in each blade. The WD-repeats of RACK1, as with all proteins that adopt this fold, overlap two adjacent propeller blades to provide an interlocking architecture. Each repeat encompasses the *d*-strand of one blade and strands *a*, *b*, and *c* of the next, terminating in the signature WD dipeptide at the end of strand *c* such that the aspartic acid (or equivalent residue) is exposed on the propeller's lower face (Adams *et al*., 2011).

The available crystal structures for Gβ and other WD-repeat proteins have established the structural basis for scaffold interactions with a range of partner proteins (Xu and Min, 2011). Unfortunately, structural information relating specifically to the interactions of RACK1 with its numerous protein partners currently remains very limited (Adams *et al*., 2011).

1.2.2 Physiological functions of RACK1

There are emerging critical roles for RACK1 in development. RACK1 has been implicated in the membrane localization of van gogh-like 2 (Vangl2) in zebrafish. The Vangl2-interacting region of RACK1 has been shown to exert a dominant-negative effect on Vangl2 localization and gastrulation (Li *et al*., 2011). The interaction between RACK1 and tyrosine-protein kinase-like 7 (PTK7) has also been shown to be a requirement for neural tube closure in *Xenopus* (Wehner *et al*., 2011). In *Arabidopsis*, where three RACK1 homologues are present,

the RACK1 gene products are essential regulators of plant development (Chen *et al*., 2006; Guo and Chen, 2008). RACK1 homologs in *Drosophila* (Kadrmas *et al*., 2007), *Aspergillus nidulans* (Hoffmann *et al*., 2000), *Schizosaccharomyces pombe* (McLeod *et al*., 2000) and *Trypanosoma brucei* (Rothberg *et al*., 2006) have similarly been shown to be central to various stages of the developmental process.

The scaffolding of signalling proteins by RACK1 at receptors is particularly important in dynamic processes such as cell migration, cell adhesion and cell spreading (Hermanto *et al*., 2002; Kiely *et al*., 2002; 2005; 2009). All of these processes require the highly regulated converging of transient signalling between receptors. For instance, RACK1 was first discovered to be a mediator of cell spreading by establishing contact with the extracellular matrix and growth factor receptors at adhesion sites (Hermanto *et al*., 2002). As a scaffold protein RACK1 plays very important roles during cell migration. Cell migration is a fundamental process required for embryonic development, wound healing and immune responses, and the components of cell migration are functionally conserved in evolution (Adams *et al*., 2011). Cell migration is a dynamic process involving multiple steps: cytoskeletal reorganisation to form leading edge protrusions, turnover of focal adhesions, generation of mechanic forces, retraction of the cell tail, and detachment from the surrounding extracellular matrix (Gandin *et al*., 2013). Evidence is mounting of RACK1 involvement in various stages of this process. RACK1 is essential for cell migration, and the protein binds to many components of the cell migration machinery including kinases, phosphatases and the cytoplasmic domains of cell surface receptors (McCahill *et al*., 2002; Sklan *et al*., 2006). RACK1 also functions in direction sensing of migrating cells. A complex of RACK1, focal adhesion kinase (FAK), and the cAMP-degrading phosphodiesterase PDE4D5 is recruited to nascent adhesions, where it modulates cell polarity (Serrels *et al*., 2010).

Some of the importance of RACK1 in disease pathology results from its ability to modulate the innate immune response and activation of interferon (IFN) signalling (Gandin *et al*., 2013). Many pathogens, especially viruses, evade the host immune response by interfering with IFN signalling. RACK1 interacts with IFNα/β receptor and recruits signal transducer and activator of transcription 1 (STAT1) into this complex, which is required for IFN signalling (Usacheva *et al*., 2001). RACK1 has been suggested to be a prognostic marker in breast cancer, and its elevated expression is associated with poor clinical outcome (Al-Reefy and Mokbel, 2010; Cao *et al*., 2010). Furthermore, RACK1 promotes proliferation, migration and metastasis of breast cancer cells in vitro and in vivo through activation of the RhoA/Rho kinase pathway (Cao *et al*., 2010; 2011). RACK1 is also implicated as a key player in ovarian cancer (Williams *et al*., 2004), prostate cancer (Hellberg *et al*., 2002), and in cancers caused by pathogens such as human papillomavirus (HPV16) (Boner and Morgan, 2002) and *Helicobacter pyroli* (Hennig *et al*., 2001).

As a primary RACK1-binding partner (Ron *et al*., 1994; 1995), PKCβII has been identified as an important factor linking ribosomal RACK1 and translational control in mammals (Gandin *et al*., 2013). RACK1 recruits active PKCβII onto ribosomes, where it phosphorylates eukaryotic initiation factor 6 (eIF6) (Sharma *et al*., 2013). This confirms the importance of RACK1-PCKβII-eIF6 complex in the regulation of translation. In addition to a positive role for ribosomal RACK1 in mRNA translation, a negative role for RACK1 in gene expression at the posttranscriptional level has been reported in yeast (Gandin *et al*., 2013). Depletion of RACK1 results in increased ribosomal activity in cell-free in vitro translation assays and a concomitant increase in protein levels in vivo (Gerbasi *et al*., 2004).

1.2.3 Post-translational Modification of RACK1

Apart from phosphorylation which appears as an important process that modulates binding of RACK1 to other proteins, not much is known about other possible post-translational modifications. There are lots of tyrosine residues in the RACK1 sequence. Phosphorylation of RACK1 by c-Abl mediates the interaction with FAK (Kiely *et al.*, 2009), while phosphorylation/dephosphorylation of RACK1 also regulates its mutually exclusive association with PP2A and β1 Integrin (Kiely *et al.*, 2008; 2006). Specifically, Src phosphorylates RACK1 on Y-228 and 246 (Chang *et al.*, 2002).

1.3 G protein-linked signalling in *Dictyostelium discoideum*

In *D. discoideum,* G protein-linked signal transduction events, in particular, are essential for chemotaxis, cell aggregation, morphogenesis, gene expression, and pattern formation (Devreotes, 1994). A major breakthrough in the identification of key proteins that are involved in eukaryotic chemotaxis was the discovery that cAMP receptors in *D. discoideum* are members of GPCRs (Klein *et al.*, 1988; Saxe *et al.*, 1988). Members of the large family of GPCRs detect various extracellular stimuli, including hormones, neurotransmitters, odorants, light, and chemoattractants (Jin *et al.*, 2008; Gilmann, 1987). Activation of GPCRs induces events which lead to dissociation of heterotrimeric G proteins into Gα and Gβγ subunits, which in turn transduce cascade of signals to intracellular signalling components that culminate cell responses. Of the 12 Gα subunits present in *D. discoideum*, some have been characterised. The Gα2 subunit is required for cellular responses to the extracellular cAMP signal that directs the aggregation process during development (Kumagai *et al.*, 1989). The Gα3 subunit is also required for aggregation but only in absence of exogenous cAMP, suggesting that Gα3 subunit is not directly involved with responses to extracellular cAMP (Brandon and Podgorski, 1997; Brandon *et al.*, 1997). Finally, the Gα4, Gα5 and Gα8

subunits play important roles in the development of spores and the anterior prestalk cells, respectively, consistent with the increased expression of these subunits upon aggregate formation (Hadwiger and Firtel, 1992; Hadwiger and Srinivasan, 1999; Hadwiger *et al.*, 1991; 1994; Wu and Janetopoulos, 2013).

1.4 *D. discoideum* G protein β subunit-null mutants

In chemotaxis, amoeboid motile cells, like leukocytes and *Dictyostelium* cells, respond directionally to chemical gradients; in phagocytosis, they bind and engulf foreign organisms or apoptotic cells (Devreotes and Zigmond, 1988; Rabinovitch, 1995). Chemotaxis and phagocytosis seem to be closely related, suggesting that the underlying signal transduction events and cytoskeletal responses have evolved in parallel (Metchnikoff, 1968). In the simple eukaryote *D. discoideum* and in amoeboid cells of the immune system of animals, chemotactic and phagocytic stimuli elicit a remarkably similar spectrum of behavioural events and biochemical reactions (Devreotes and Zigmond, 1988; Greenberg, 1995). Foremost among these is the polymerization of actin into filaments that support the extension of pseudopods and the formation of phagocytic cups (McRobbie and Newell, 1993; Greenberg, 1995; Zigmond, 1996). Chemotaxis and phagocytosis involve both G protein-coupled and tyrosine kinase-linked signal transduction pathways (Parent and Devreotes, 1996, Murphy, 1996). With regard to phagocytosis, bound particles activate protein tyrosine kinases, such as *syk*, leading to actin polymerization and rearrangement, possibly through involvement of the small G protein Rho (Greenberg *et al*., 1994, 1996; Indik *et al*., 1995; Hackam *et al*., 1997). Heterotrimeric G proteins have been involved in chemotactic activation of macrophages, which leads to phagocytosis (Thelen and Wirthmueller, 1994), and in phagosome-endosome fusion (Desjardins *et al*., 1994)

D. discoideum amoebae contain a single G protein β subunit; its deletion creates cells that lack functional G proteins (Lilly *et al.*, 1993; Wu *et al.*, 1995). These mutants are severely defective in chemotaxis, aggregation, and development. When plated on bacterial lawns, they form smooth plaques consisting of monolayers of undifferentiated cells. These plaques are much smaller than those of wild type (Wu *et al.*, 1995). This slow growth reflects a severe defect in phagocytosis, which is primarily due to a failure in organizing the actin meshwork into a phagocytic cup (Peracino *et al.*, 1998).

1.5 Aim of this study

The aim of this study was to characterise the *D. discoideum* RACK1 (DdRACK1) protein which we initially identified as a binding partner of Receptor phosphatidylinositol kinase A (RpkA), an unusual GPCR which functions in phagocytosis and antibacterial defense in *D. discoideum* (Riyahi *et al.*, 2011). DdRACK1 was characterised based on the following features:

1. Its structure and homology to other RACK1 species

2. Subcellular localisation and its dimerization properties

3. Its ability to interact with G proteins

The final part of this study was focused on the analyses of the effect of *D. discoideum* RACK1 (DdRACK1) overexpression during growth and development.

2 Materials and Methods

Standard molecular biology techniques were performed as described in Sambrook et al., 1989. Light and heavy instruments used were properties of the Department. Standard laboratory materials and reagents were obtained from local suppliers.

2.1 Kits

NucleoSpin Extract 2 in 1	Macherey-Nagel
Quick Change Site-Directed Mutagenesis kit	Stratagene
pGEM-Teasy Vector kit	Promega
Pure Yield Plasmid System	Promega
VIVASPIN 500	sartoriusstedim biotech

2.2 Enzymes, antibodies and antibiotics

2.2.1 Enzymes for molecular biology:

Taq-polymerase	Promega
T4 DNA ligase	Boehringer
Thrombin	GE Healthcare
Pfu DNA polymerase	Promega
Alkaline phosphatase	Roche
Restriction endonuclease	New England Biolabs
Ribonuclease A	Sigma

2.2.2 Antibodies

2.2.2.1 Primary antibodies

Mouse monoclonal anti- α-actinin mAb 47-16-8	(Schleicher *et al.*, 1984)
Mouse monoclonal anti-csA mAb 33-294	(Berthold *et al.*, 1985)
Mouse monoclonal anti-cap32 mAb 188-19-95	(Haus *et al.*, 1993)
Mouse monoclonal mAb 5E7	(Fendly *et al.*, 1990)
Mouse monoclonal anti-GFP mAb K3-184-2	(Noegel *et al.*, 2004)
Monoclonal antibody anti-mRFPmars mAb K73-875-7	(Fischer *et al.*, 2004; Omosigho *et al.*, submitted)
Rabbit polyclonal anti-GST	(Xiong *et al.*, 2008)
Rabbit polyclonal anti-RACK1	(This study)

2.2.2.2 Secondary antibodies

Goat anti-mouse IgG, peroxidase conjugated	Sigma
Goat anti-rabbit IgG, peroxidase conjugated	Sigma

2.2.3 Antibiotics

Ampicillin	Gruenenthal
Blasticidin S	Biomedicals
Dihydrostreptomycinsulphate	Sigma

Tetracyclin	Sigma
Gentamicin	Sigma
G418	Sigma

2.3 Media and Buffers

All solutions and media used in the course of these experiments were prepared with deionized water from a pure water source in the laboratory. All other buffers and solutions which were not mentioned here are seen in the methods.

2.3.1 Buffers and Solutions

GST-fusion protein elution buffer, pH 7.2

50 mM Tris/HCl, pH 7.2

100 mM NaCl

10 mM reduced glutathione

0.2 % Tween-20

10 mM DTT

10 x NCP-buffer, pH 8.0

12.1 g Tris

87.0 g NaCl

5.0 ml Tween 20

2.0 g NaCl

Made up to 1 litre with deionised water

50 x Tris/Acetate buffer (TAE), pH 8.0

242 g Tris

57.1 ml 16.6 M Glacial acetic acid

100 ml 0.5 M EDTA

Made up to 1 litre with deionized water

TBS lysis buffer, pH 7.2

50 mM Tris/HCl, pH 7.2

100 mM NaCl

TE-buffer, pH 8.0

10 mM Tris/HCl, pH 8.0

1 mM EDTA

TBS-T buffer, pH 7.2

50 mM Tris/HCl, pH 7.2

100 mM NaCl

0.2 % Tween-20

2.3.2 Bacteria medium and agar plates

LB-Medium (Sambroock *et al*., 1989)

10 g/L Bacto-Tryptone

5 g/L Yeast Extract

5 g/L NaCl

pH was adjusted to 7.0 with 5 M NaOH and made up to 1 litre with deionised water and then autoclaved.

Ampicillin-LB-agar Plates

For the preparation of LB-agar plates, 0.9% (w/v) agar was added to LB-medium. After autoclaving and cooling to 55°C, 100 mg/l ampicillin was added. Stroage was at 4°C.

To prepare plates with ampicillin/IPTG/X-Gal, 10 µl of 1M IPTG and 50 µl of 20 mg/ml X-Gal was spread over the surface of LB-Amp-Plates and allowed to absorb for at least 30 minutes at 37°C prior to use.

2.3.3 Yeast medium

YEPD-Medium

20 g Difco-Peptone

10 g Yeast Extract Made up to 1 litre with deionized water and then autoclaved

YEPD-Agarplates

20 g Difco-Peptone

10 g Yeast Extract

18 g Agar-Agar

Made up to 1 litre and then autoclaved

100x L-Adenine solution

200 mg of L-Adenine dissolved in 100 ml H_2O. Dissolution was aided with addition of drops of HCl and then sterile filtered.

100x L-Tyrosine solution

300 mg of L-Tyrosine dissolved in 100 ml H_2O. Dissolution was aided with addition of drops of NaOH and then sterile filtered.

100x L-Histidine solution

200 mg of L-Histidine dissolved in 100 ml H_2O and then sterile filtered.

100x L-Leucine solution

200 mg of L-Leucine dissolved in 100 ml H_2O and then sterile filtered.

100x L-Tryptophan solution

200 mg of L-Tryptophan dissolved in 100 ml H_2O and then sterile filtered.

100x L-Uracil solution

200 mg of L-Uracil dissolved in 100 ml H_2O. Dissolution was aided by slightly warming solution before it was sterile filtered.

50x Drop-out Solution

1,500 mg Isoleucine

7,500 mg Valine

1,000 mg Arginine

1,500 mg Lysine

1000 mg Methionine

2,500 mg Phenylalanine

10,000 mg Threonine

Filled up to 1 litre and then sterile filtered

20x Drop-out Solution

20 ml 50x Drop-out solution

10 ml 100x L-Uracil

10 ml 100x L-Tyrosine

10 ml 100x L-Adenine

1 M 3-Amino-1, 2, 4-triazol solution (3AT)

8.4 g 3-Amino-1, 2, 4-triazol dissolved in 100 ml deionised H_2O and then sterile filtered.

Composition of the Yeast Selection Plates and Selection Medium

	SD/-Leu/-Trp	**SD/-Leu/-Trp/ -His/+3AT**
Yeast Nitrogen Base (g)	6.7	6.7
Agar-Agar (g)	20	20
Water (ml)	770	745
20% Glucose solution (ml)	100	100
20x Drop-out solution (ml)	50	50
100 x L-Histidine (ml)	10	
100 x L-Leucine (ml)	-	
100 x L-Tryptophan (ml)	-	
1M 3AT Solution (ml)		25

Yeast selection media were prepared but without the addition of the 20 g Agar to the preparations. To test for protein interactions, transformants on the SD /-Leu /-Trp plates were transferred to the SD /-Leu /-Trp /-His /+3AT plates. After 6-8 days, the colonies which grew were used to perform β-galactosidase activity staining.

2.3.4 Media and buffers for *Dictyostelium* cultures

AX2-Medium, pH 6.7 (Claviez *et al*., 1982)

7.15 g Yeast extract

14.3 g Peptone

18.0 g Maltose

0.486 g KH_2PO_4

0.616 g Na_2HPO2 x H_2O

Made up with H_2O to 1 Liter

Soerensen-phosphate buffer, pH 6.0 (Malchow *et al.*, 1972)

2 mM Na_2HPO_4

14.6 mM KH_2PO_4

Phosphate-Agar plates, pH 6.0

9 g Agar made up with Soerensen-phosphate buffer pH 6.0 to 1 Litre

Water agar plates

1% Agar in Water

2.3.5 Bacteria, *D. discoideum*, and yeast strains

Bacteria Cell Stock

E.coli (XL1 blue) (Bullock *et al.*, 1987)

E.coli (BL21) (Studier and Moffat, 1986)

D. discoideum

AX2-214, also known as AX2 is a derivative of NC4 wild isolate (Raper, 1935) which can grow axenically.

Yeast strain

Saccharomyces cerevisiae Y190 (*His3* and *lacZ* reporter genes) (Johnston *et al.*, 1991; Harper *et al.*, 1993).

2.3.6 Oligonucleotides

Oligonucleotides used for PCR (Polymerase Chain Reaction) were purchased from Sigma-Genosys in Steinheim.

RACK1

RACK1_fw GGATCCATGGAACAACAAAAAGCACCAC

RACK1_rev ATCGATTTATTGGGATGATGATTTGTAAAC

Gβ subunit

Gβ_fw ATCGCGGATCCATGTCATCAGATATTTCAGAAAAAATT

Gβ_rev ATCGCCTGCAGTTAAGCCCAAATCTTGAGGAGAGAATC

Gγ subunit

Gγ_fw GCGGGATCCACCATGGAAATGTCCGAATCACAATTAAAAAAAGTT

Gγ_rev ATCGCGGATCCTTATAACACAGAACATCCATTTCCTTT

Gα subunits

Gα1_fw ATCGCGGATCCATGGGTAATATTTGTGGTAAACCA

Gα1_rev ATCGCCTCGAGTTAAAGAATCATACCAGCTTCACCCAA

Gα2_fw ATCGCGAATTCATGGGTATTTGTGCATCATCAATG

Gα2_rev ATCGCAGATCTTTAGGATCCAGAATATAAACCAGCTTTCATAACACA

Gα3_fw ATCGCCTCGAGATGGATTTCAATCCAGTACCACCA

Gα3_rev ATCGCAGATCTTTAACAATAAAATTCTAAAGTTTTTGAAATTAT

Gα4_fw ATCGCGAATTCATGAGATTCAAGTGTTTTGGATCA

Gα4_rev ATCGCCTCGAGTTAGGATCCGAAGTGTTCTAAAGCTTGAGATAA

Gα5_fw ATCGCGAATTCATGGGTTGTATATTAACAATTGAAGCA

Gα5_rev ATCGCAGATCTTTAGGATCCATAATTTATGATTGTATTAAAGATATTTTT

Gα6_fw ATCGCGAATTCATGGCATTTTTATGTAAATCAAATGAT

Gα6_rev ATCGCAGATCTTTAGGATCCAACAAGATCCATAACATGACCAAC

Gα7_fw ATCGCGAATTCATGAGTAGCACTACAACAAATACA

Gα7_rev ATCGCAGATCTTTAGGATCCGATACCTCCTTCCTCCATAGTTTG

Gα8_fw	ATCGCGGATCCATGGGTTGCTATCAATCACGTGTT
Gα8_rev	ATCGCCTCGAGTTAAGAATTAATTTTGGCGGTTGCACC
Gα9_fw	ATCGCGAATTCATGGGTTGTAATTCAAGTAGTGAA
Gα9_rev	ATCGCAGATCTTTAGGATCCATAGTGAAGTTTTAAGATAGATTGAAT
Gα10_fw	ATCGCGAATTCATGTCATTTTTATGCTCAGAAAATTCA
Gα10_rev	ATCGCAGATCTTTAGGATCCATTTTTTACAGAATTATATGTTATGTT
Gα11_fw	ATCGCGAATTCATGGGAAGCCAATTTAGTGTTTTA
Gα11_rev	ATCGCAGATCTTTAGGATCCAATTGTATCTTCTAATATTTTTTTAAC
Gα12_rev	ATCGCGGATCCATGTGTACAAGAAATAAAAAAGATATT
Gα12_rev	ATCGCCTCGAGTTATAAAATTTCTGCAACATTCATTAATAA

Primers for Site-Directed Mutagenesis

mut_fw	CCAGAAGTCAAAGAACAAGCTTTCGACTCAGAGGAAGAGGAA GAATCAAAACCAAAGCACC
mut_rev	GGTGCTTTTGGTTTTGATTCTTCCTCTTCCTCTGAGTCGAAAGCT TGTTCTTTGACTTCTGG

2.4 Methods

2.4.1 Growth, development and transfection

Cells were either grown on a lawn of *K. aerogenes* on SM agar plates, on a lawn of *E. coli* B12 on NA-agar or cultivated in shaking suspension (160 rpm) or in a submerged culture at 21-23°C in axenic medium (Harloff *et al*., 1989). Development was initiated by plating 5 x 10^7 cells which were washed twice with Soerensen phosphate buffer (17 mM Na^+/K^+ phosphate, pH 6.0) on phosphate agar plates and monitored. Development was also followed for cells starved in Soerensen phosphate buffer in shaken suspension (1 x 10^7 cells/ml; 160 rpm at 22ºC) or in petri dishes. Mutants were maintained in the presence of appropriate antibiotics (2-4 µg/ml G418) (Roche Applied Science) or (3-5 µg/ml Blasticidin) (MP Biomedicals Inc., Eschwege, Germany). The following strains have been used; AX2-214 (wild type) (Noegel *et al*., 1985), AX2 expressing GFP-, YFP- or RFP-tagged fusion proteins, Gβ null mutants LW6 (Wu *et al*., 1995; Peracino et al., 1998) and LW6 expressing GFP-DdRACK1. The corresponding plasmids were introduced by electroporation using a Biorad electroporator Gene Pulser Xcell (Biorad, München, Germany) according to the protocol supplied.

2.4.2 Cloning of RACK1 cDNA and expression of recombinant proteins

For expression of recombinant *D. discoideum* RACK1 and Gα subunits as glutathione S transferase (GST) fusion proteins in *E. coli*, full-length cDNAs were respectively cloned into pGEX-4T-1 vector (GE Healthcare Life Sciences). *E. coli* strain XL1 Blue was used for expression of the GST fusion proteins. Induction of protein expression was with 0.25 mM isopropyl β-D-thio-galactoside (IPTG) when an OD_{600} of 0.8 was reached. Cells were further cultured at 30ºC for 3 hours. They were harvested, lysed in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, supplemented with Protease inhibitors (0.5 mM PMSF, 1mM Benzamidine and

Complete (Roche) and 1mM DTT with an EmulsiFlex cell homogenizer (Avestin Europe GmbH, Mannheim, Germany). Lysates were separated into soluble and insoluble fractions by centrifugation at 18,000g. The fusion proteins from the soluble fraction were purified using GST-Sepharose beads (GE Healthcare).

For cleavage of proteins from GST-Sepharose beads, the GST fusion proteins were washed 5 times with cleavage buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.2 % Sarcosyl). Beads were then re-suspended in cleavage buffer and 3-10 U thrombin/mg fusion protein were added to the beads and incubated with little agitation at room temperature overnight. As RACK1 was released from the beads together with some GST, we next performed an anion exchange chromatography step in order to separate the proteins. For this the protein solution was dialyzed against 20 mM Tris/HCl, pH 8.0, and 1 mM EDTA overnight before loading onto a DE-52 Sephadex column which had been calibrated with 50 mM Tris/HCl, pH 8.0, 1 mM EDTA. The protein was eluted with 1 M NaCl and the eluate dialyzed and analyzed by SDS-PAGE.

For expression in AX2 the cDNA was cloned into pBsr-N2-GFP vector and expressed as GFP-RACK1 (GFP N-terminal) under control of the actin 15 promoter and also into mRFPmars plasmid for RFP-RACK1 (RFP N-terminal) (Blau-Wasser et al., 2009; Fischer *et al*., 2004). A PCR-mediated site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) was used to generate mutations in the GST-RACK1 and GFP-RACK1 plasmids. The mutations were confirmed by sequencing.

2.4.3 Phosphoinositide binding assay

PIP-strips supplied by Echelon Biosciences, Inc. (Salt Lake City, Utah, USA) were used to perform phosphoinositide binding according to the supplied protocol. Briefly, GST and GST-fusion proteins were eluted from the glutathione agarose beads with elution buffer (20 mM

reduced glutathione, 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.2% Tween-20, and 100 mM DTT).

The membranes were blocked with 0.1% ovalbumin (Sigma # A-5253) in TBS for one hour at room temperature. After discarding the blocking solution membranes were incubated with 1 mg/ml GST-fusion proteins in TBS-T (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.2 % Tween-20) at room temperature for one hour. The protein solution was then discarded and the membranes were washed with TBS-T three times 10 minutes each. Bound protein was detected by western blot analysis with GST polyclonal antibodies as primary and anti-rabbit IgG-peroxidase (Sigma # A-6154) as secondary antibody followed by enhanced chemiluminescence.

2.4.4 Lipid vesicle preparation and sedimentation assay

Phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3 were obtained from Sigma and disolved in chloroform. Liposome binding experiments were performed with a modified published liposome binding assay protocol (Blume *et al*., 2007). Lipid mixtures containing 65% PC, 20% PE, 5% PS and 10% individual phosphoinositides were produced by mixing appropriate lipid solutions in chloroform/methanol. Slow flow nitrogen gas was used for the production of a film on the glass and vacuum desiccation for 30 min for solvent removal. Sterile-filtered sucrose binding buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 1 mM EDTA, 0.1 M sucrose) was added to a final lipid concentration of 1 mg/ml and incubated at 37ºC for 2 h. Lipids were then sonicated in a waterbath-sonicator for 10 sec. To test liposome binding, a 100 µl reaction mixture of freshly prepared liposomes and 5 µg of purified protein were incubated for 15 min at room temperature and centrifuged at 100,000 x g (42,000 rpm) at 4ºC for 25 min in a Beckman table top ultracentrifuge Optima TLX (TLA 45 rotor). The supernatant was saved, and the pellet was resuspended in 100 µl of sucrose binding buffer.

Both fractions were then analyzed by SDS-PAGE followed by Coomassie Blue staining. ImageJ was used for quantification.

2.4.5 Yeast Two-Hybrid Interaction

For the yeast two-hybrid screen, the full-length cDNAs of *D. discoideum* G protein β-, γ-, α1-, α2-, α4-, α5-, α6-, α7-, α8-, α9-, α10-, α11- and α12-subunits were cloned in frame into the yeast pAS2-1 vector (Clontech), respectively, resulting in fusion to the GAL4-DNA-BD (BD, binding domain). Full-length cDNA of DdRACK1 was cloned into the yeast pACT2 vector (Clontech) resulting in a fusion to the GAL4-DNA-AD (AD, activation domain). Yeast Y190 strain which has *His3* and *lacZ* reporter genes was used for this assay.

Candidate colonies expressing interacting proteins were screened by plating on SD/-Leu/-Trp/-His/+3AT plates after which membrane colonies-lift β-galactosidase activity assay was performed according to the MATCHMAKER Y2H system manual. Briefly, colonies on SD/-Leu/-Trp/-His/+3AT selection plates were transferred to a Nitrocellulose membrane (Protran BA 85) by placing the membrane over colonies on selection plates for 20 min. The filter was carefully lifted off the agar plates and transferred (with colonies facing up) to a pool of liquid nitrogen for 10 sec. The frozen filter was then allowed to thaw at RT and placed on a Whatman filter paper presoaked in freshly prepared X-Gal solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 50 mM β-mercaptoethanol, X-Gal (1 mg/ml final concentration)) and incubated at 30°C and checked between 1 to 6 h for the appearance of blue colonies.

2.4.6 Pull down and immunoprecipitation assays

For pull down and immunoprecipitation experiments *D. discoideum* cells were lysed in 50 mM (10 mM for immunoprecipitation assay) Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% NP40, supplemented with protease inhibitor cocktail (Sigma), 0.5 mM PMSF, 0.5 mM EDTA, and 1 mM Benzamidine by passing them through a 25G syringe (10-20 strokes) and incubated with

agitation (1000 rpm) for 15 min at 4℃ (to ensure complete cell lysis) followed by a centrifugation step at 16,000 rpm for 10 min. The supernatants were either incubated with GST and GST-fusion proteins respectively or with GFP-trap beads (ChromoTek, Martinsried, Germany). After incubation for 3 h while GST beads were washed three times with wash buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, protease inhibitor cocktail, 0.5 mM PMSF, 0.5 mM EDTA, 1 mM Benzamidine), GFP-trap beads were washed with a different wash buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl, protease inhibitor cocktail, 0.5 mM PMSF, 0.5 mM EDTA, 1 mM Benzamidine). The beads were resuspended in SDS sample buffer, incubated at 95℃ for 5 min and the proteins separated by SDS-PAGE and analyzed by western blot. The Gβ and Gγ subunits used in this study were previously cloned into GFP (GFP N-terminal) and YFP (YFP C-terminal) vectors, respectively (Jin *et al.*, 2000; Zhang *et al.*, 2001).

2.4.7 In vitro cross-link assay

Purified DdRACK1 was used for a multimerization experiment as was previously described (Xiong *et al.*, 2008). Briefly, 5-10 µg/100 µl of RACK1 in 1 x PBS, pH 7.4, was incubated at room temperature in the presence of 0.001 % (v/v) glutaraldehyde for various time points. The reaction was stopped by addition of glycine to a final concentration of 0.1 M after 5, 10 and 20 min, respectively. Samples were analyzed by SDS-PAGE and western blot.

2.4.8 Test for presence of phosphotyrosine in DdRACK1

Samples from immunoprecipitation experiments from GFP-DdRACK1 bound to GFP-trap beads in the presence or absence of phosphatase inhibitors were analyzed by western blots and probed with anti-phosphotyrosine monoclonal antibody (5E7) (Fendly *et al.*, 1990).

2.4.9 Immunofluorescence analysis and life cell imaging

Immunofluorescence study was performed as previously described (Blau-Wasser *et al.*, 2009). Briefly, cells were transferred onto coverslips in Petri dishes and fixed by ice-cold methanol

(5 min, 20 °C). Cells were treated twice for 15 min (room temperature) with blocking solution (1x PBS containing 0.5% (wt/vol) BSA and 0.1% (vol/vol) fish gelatin). The appropriate antibodies were diluted in the blocking solution and applied on the cells for 1 h at room temperature; the excess of antibodies was removed by washing with the blocking solution before the 1 h incubation with the corresponding secondary antibodies. For live cell study, cells were placed in 35 mm Petri dishes (ibidi GmbH-Martinsried, Germany) and allowed to adhere to the surface. Analysis of fixed and live cells was done by laser scanning confocal microscopy using a Leica TCS SPS microscope.

2.5 Cell migration studies

This analysis was done as previously described (Blau-Wasser *et al*., 2009; Müller *et al*., 2013). Briefly, cells were plated after ~ 6 h of starvation in a chamber (ibidi GmbH-Martinsried, Germany) and migration towards aggregation centers were followed. Images were recorded at intervals of 6 s using a Leica DM-IL inverse microscope (Deerfield, IL; 40x objective) and a conventional CCD video camera and analyzed using Dynamic Image Analysis Software (DIAS, Soll Technologies, Iowa City, IA).

2.5.1 Miscellaneous methods

Cell fractionation of AX2 cells was done as described (Müller *et al*., 2013). For generation of rabbit polyclonal antibodies against DdRACK1, the GST-part of GST-DdRACK1 was removed by thrombin cleavage and DdRACK1 was used to immunize rabbits (Pineda, Berlin, Germany). The antibodies specifically recognized the bacterially produced recombinant protein, the RFP- and GFP-tagged fusion proteins as well as the endogenous protein in western blots of whole cell lysates; they were used in immunoprecipitation experiments whereas they were not suitable for immunofluorescence studies.

Protein sequences of RACK1 proteins from *H. sapiens* (P63244), *D. melanogaster* (O18640), *A. thaliana* (O24456), *S. cerevisiae* (P38011), and *D. discoideum* (P46800) were retrieved

from Uniprot protein database and aligned using ClustalW program with Blosum 62 matrix. The aligned sequences were processed through EsPript for representation. The structural coordinates of *S. cerevisiae* RACK1 (Asc1p) was obtained from protein databank (PBD: 3FRX) (Figure 4) and used as a template for modelling *D. discoideum* RACK1. MODELLER v9 was used to generate DdRACK1 model. Structures in Figures 3B and C were generated with the aid of the molecular visualization software PyMOL.

3 Results

3.1 Characterization of DdRACK1

gpbB (DDB_G0275045) is located on chromosome 2 of the *D. discoideum* genome and has 2 exons. The open reading frame encompasses 1136 bp which encodes a protein of 329 amino acids migrating as a 36 kDa protein on SDS polyacrylamide gels. Blast results showed that GpbB is highly related to the RACK1 family of proteins and the alignment of RACK1 sequences from diverse organisms such as *H. sapiens, D. melanogaster, A. thaliana, D. discoideum* and *S. cerevisiae* revealed significant sequence identity. The greatest difference is observed between propeller blades 6 and 7 where an extended loop of mainly basic amino acids is present in the *D. discoideum* protein and the *A. thaliana* RACK1 (Figure 3).

Gβ was the first WD-repeat protein to be characterized by X-ray crystallography (Wall *et al.*, 1995). Since then various other crystal structures have been reported for WD-repeat proteins (Lambright *et al.*, 1996; Sondek *et al.*, 1996) which include the recently determined structures for several RACK1 proteins, RACK1A from *A. thaliana*, Asc1p from *S. cerevisiae*, RACK1 from *T. thermophila* and RACK1 from human (Coyle *et al.*, 2009; Rabl *et al.*, 2011; Ruiz *et al.*, 2012; Ullah *et al.*, 2008; Yatime *et al.*, 2011). These structural studies confirmed the seven-bladed β-propeller structure. In the RACK1 structure each propeller blade consists of a four-stranded antiparallel β-sheet, where strand A lines the central canal of the protein, and strand D is present on the outer circumference. Adjacent blades are connected by a loop bridging from strand D on one blade to strand A on the next. These loops are exposed on the top face of the propeller blade as are the β-turns linking strands B and C in each blade. The loops connecting strand A to B and strand C to D in each blade are located on the reverse, slightly larger face of the propeller (Adams *et al.*, 2011).

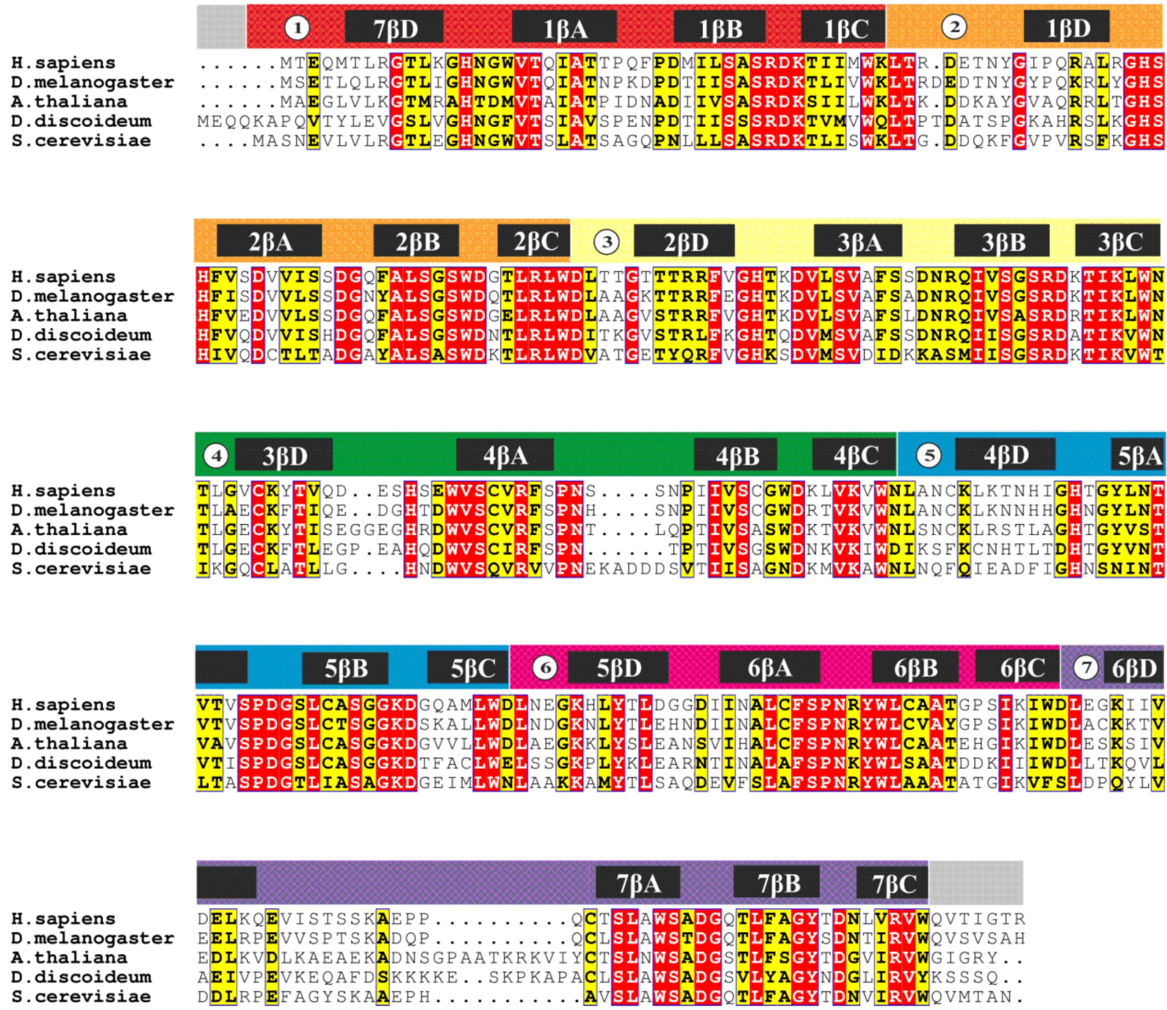
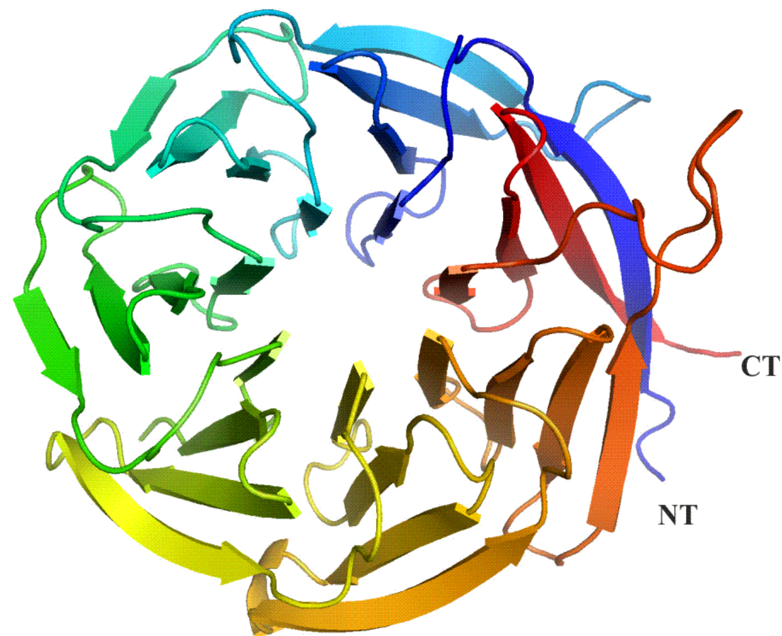


Figure 3 Sequence alignment of RACK1 orthologues and their UniProt accession numbers from *H. sapiens* (P63244), *D. melanogaster* (O18640), *A. thaliana* (O24456), *D. discoideum* (P46800) and *S. cerevisiae* (P38011). The WD40 repeats and β-propeller blade positions are written above the sequences. Alignment was done with the ClustalW program and processed through ESPript for representation. All conserved residues are shown in red and similar residues in yellow

Most notably, the D-A loop between blades 6 and 7 in the RACK1 species is 8 to 19 residues longer than the cognate region of $G\beta_1$ and forms a knob-like projection from the upper face of

the propeller (discovered in the crystal structure of *A. thaliana* RACK1A) (Adams *et al.*, 2011). This sequence is quite unusual in the *D. discoideum* protein as it is rich in lysine residues. These general features described for RACK1 proteins are also present in DdRACK1 when we modelled the DdRACK1 sequence to the crystal structure of *S. cerevisiae* RACK1 (Asc1p) which reveals a comparable structure (Figure 4, 5).



S. cervesiae RACK1 (3FRX)

Figure 4 Ribbon diagram of *S. cerevisiae* RACK1 (Asc1p) (PDB: 3FRX) which was used as template for DdRACK1 model. Shown are the seven β-propeller blades. Coordinates were retrieved from protein data bank (PDB) (www.rcsb.org) and modelled with the aid of MODELLER 9 program and visualized by the software PyMOL.

The DdRACK1 protein harbors in the knob-like projection in the D-A loop between blades 6 and 7 of sequence a polybasic stretch with four lysine residues. These residues were changed

to glutamic acid and the surface properties of this region viewed alongside the wild type protein during the modelling of DdRACK1.

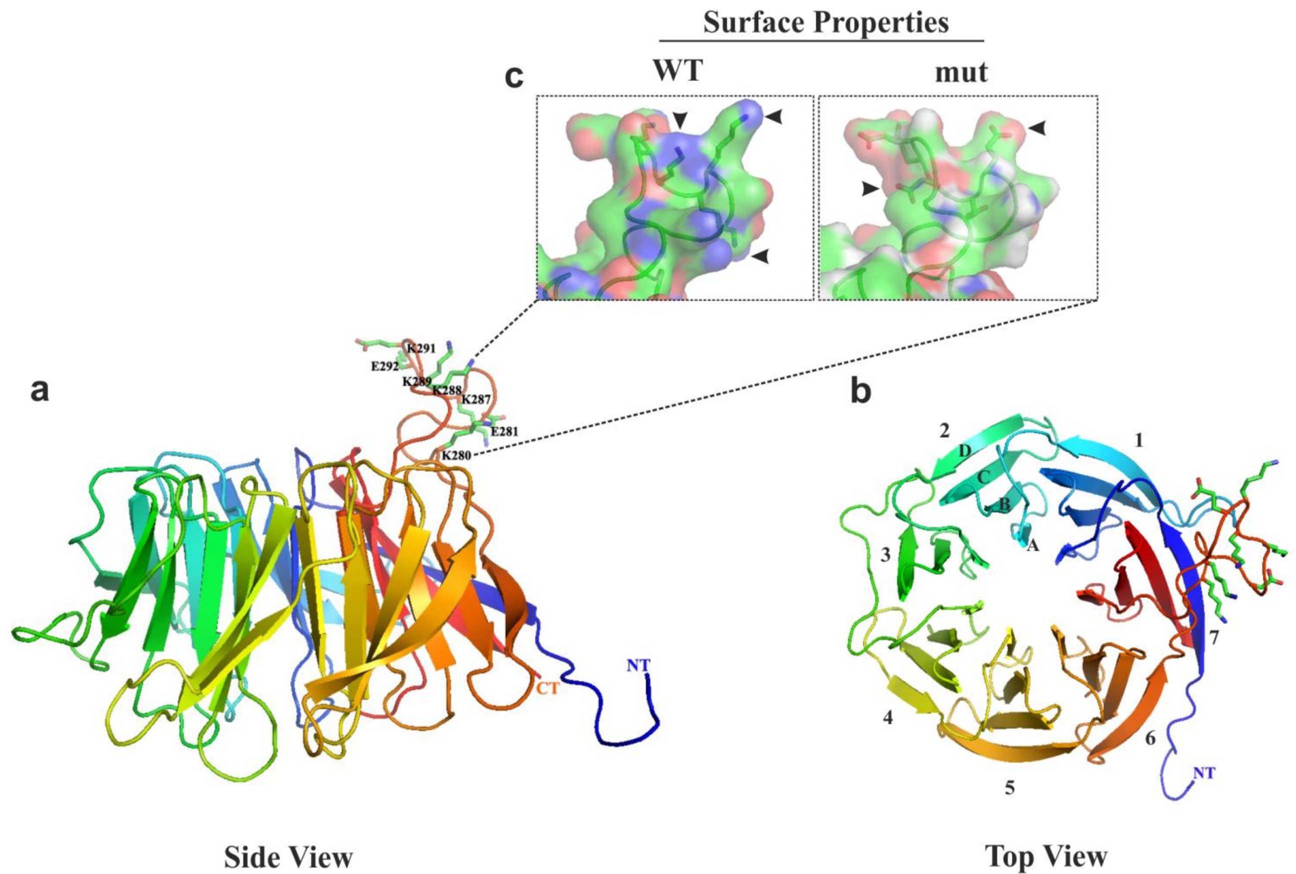


Figure 5 Ribbon diagram of DdRACK1 modelled with the aid of MODELLER9 program, with yeast RACK1, which was retrieved from protein data bank (PDB) (www.rcsb.org), as template. Structures were finally generated using molecular visualization software PyMOL. In (c) the surface charge of the extension after mutation of the basic residues into glutamic acid (see 3.6) is shown.

3.2 Subcellular localization of RFP-/GFP-DdRACK1

When we expressed RFP-DdRACK1 in AX2 cells expressing the G protein beta-subunit as GFP-fusion protein for labelling the plasma membrane, we found that RFP-DdRACK1 is present throughout the cytosol (Figure 6).

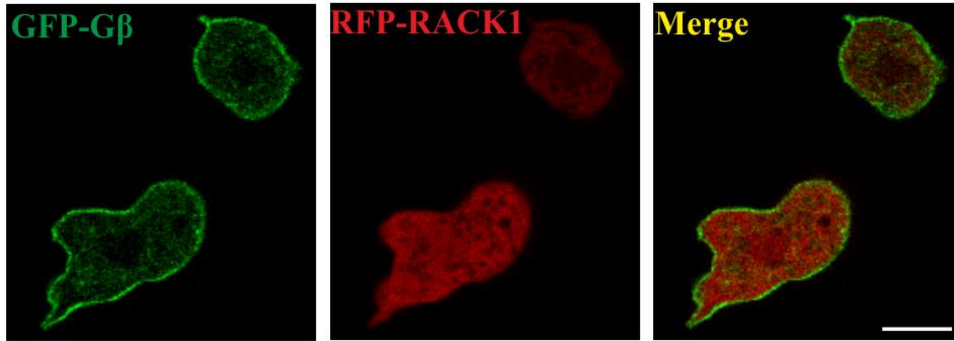


Figure 6 Subcellular localization of DdRACK1. To determine the localization of DdRACK1, AX2 wild type cells co-expressing GFP-Gβ and RFP-DdRACK1 were used to perform confocal live cell microscopy. Localization of RFP-DdRACK1 is mainly in the cytosol and GFP-Gβ is distinctly at the plasma membrane. Scale bar, 5 µm.

In live cell confocal imaging analysis, RFP-DdRACK1 co-localized with GFP-Gβ at the front of newly formed membrane protrusions (Figure 7, arrow).

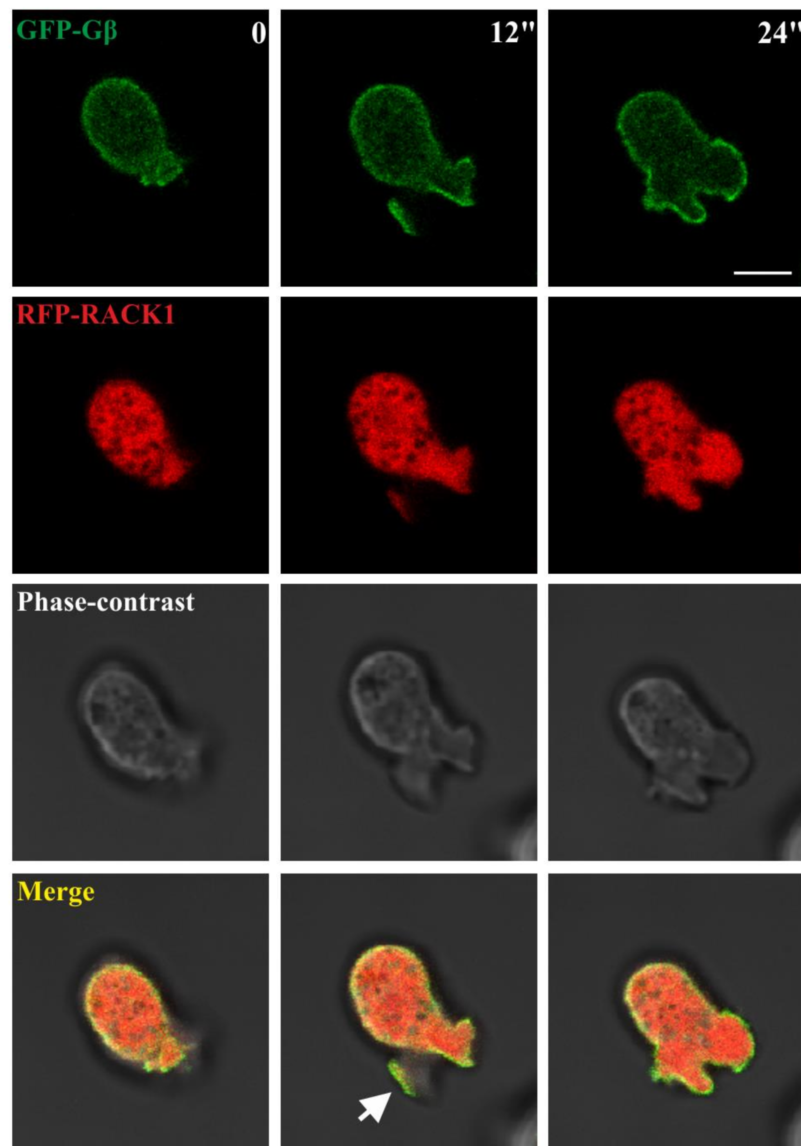


Figure 7 DdRACK1 co-localisation with Gβ. Representative images of a series show co-localization of RFP-DdRACK1 with GFP-Gβ at membrane protrusions (arrow) formed after 12 sec of imaging, which disappeared after 24 sec. Scale bar, 5 µm.

Close inspection of AX2 cells expressing GFP-DdRACK1 revealed that newly formed protrusions initially were devoid of RACK1 but were later filled with GFP-DdRACK1 after several seconds (Figure 8, arrow).

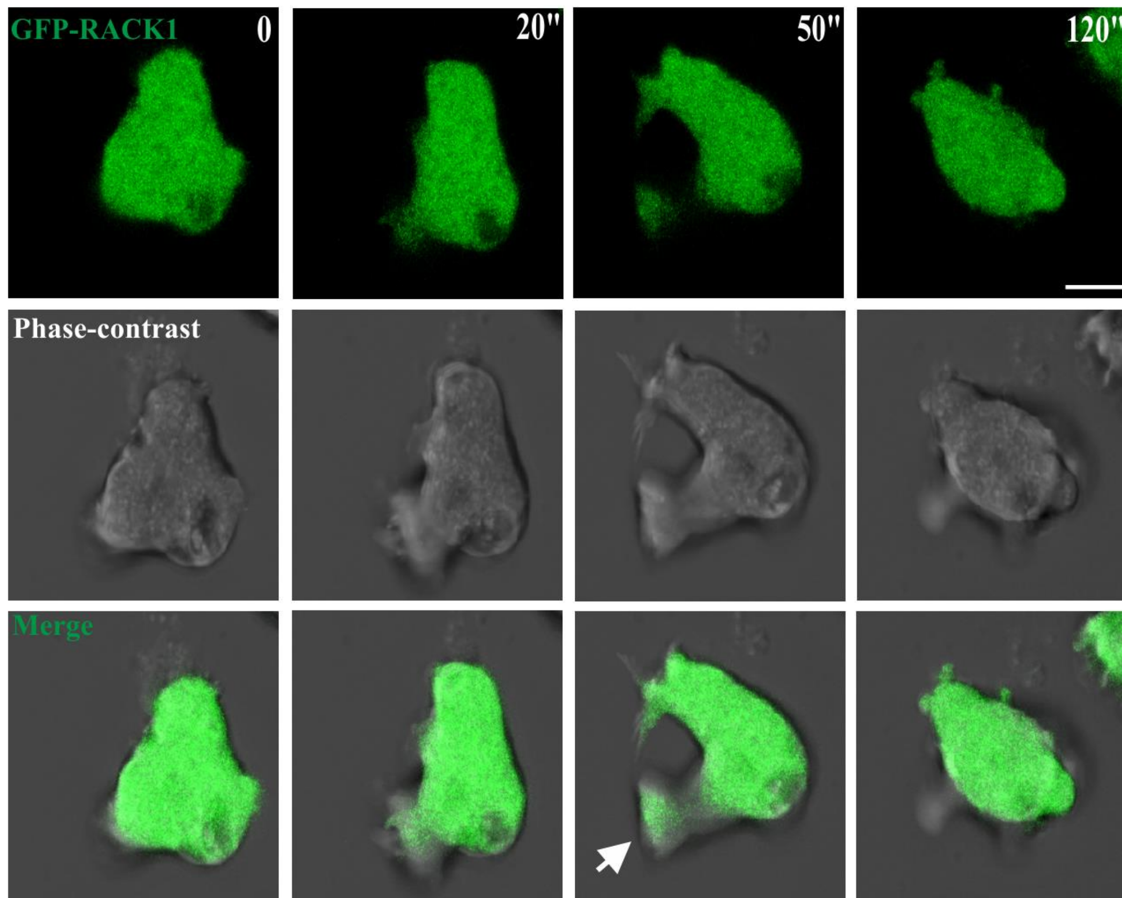


Figure 8 Confocal live cell images of GFP-DdRACK1/AX2 cells. Like RFP-DdRACK1 in Figure 7 GFP-DdRACK1 filled membrane protrusions after 50 sec of live imaging which disappeared after 120 sec. Scale bar, 5 µm.

We also generated a mutant protein in which the four lysine residues in the extended loop of RACK1 (see Figure 5) were exchanged with glutamic acid residues. Analysis of GFP-DdRACK1mut cellular distribution showed similar localization like GFP-DdRACK1 (Figure 9).

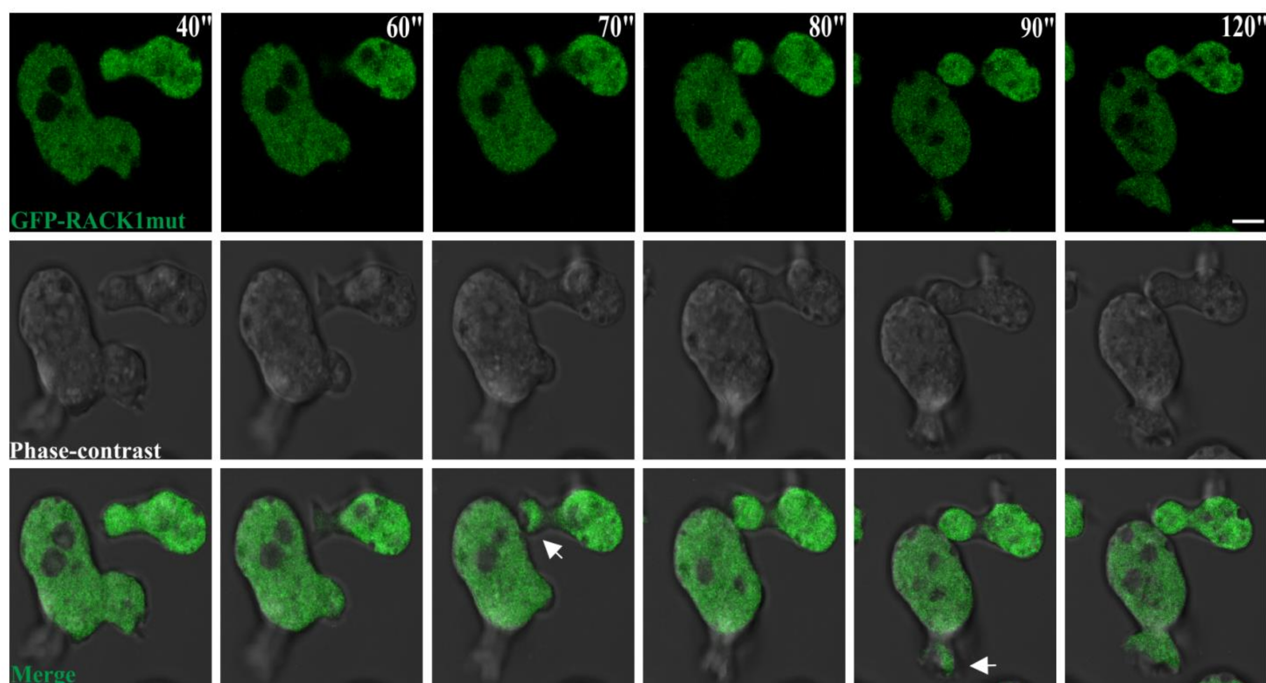


Figure 9 Subcellular localization of DdRACK1mut. Imaging of GFP-DdRACK1mut/AX2 cells showed localization of GFP-DdRACK1mut at membrane protrusions after 70 sec and 90 sec (arrows) respectively. Scale bar, 5 µm.

3.3 Subcellular distribution of DdRACK1, GFP-DdRACK1 and DdRACK1mut, and the developmental expression pattern of DdRACK1

Cell fractionation experiments were performed to further study the subcellular distribution of endogenous DdRACK1 as well as its GFP-fusion constructs. While reasonable amounts of endogenous DdRACK1 associated with the pellet fractions, only faint bands of both GFP-DdRACK1 and GFP-RACK1mut were seen in the pellet fractions (Figure 10). These low GFP-fusion protein amounts in the pellet fractions may be explained by their moderately low level of overexpression with respect to endogenous DdRACK1. α-Actinin which served as cytosolic marker was exclusively present in the cytosolic fraction. A membrane association of RACK1 is not surprising as it has been repeatedly found in phagosomal preparations from mouse and *Drosophila*, and GpbB has been found in phagosomal preparations from *D.*

discoideum (Boulais *et al*., 2010; Gotthardt *et al*., 2006; Rogers and Foster, 2007; Urwyler *et al*., 2009).

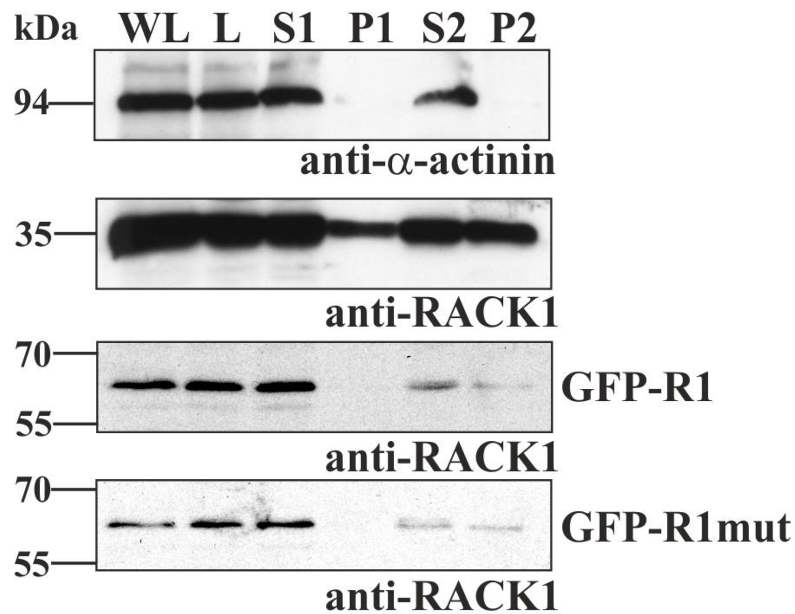


Figure 10 Subcellular fractionation of AX2 and AX2 expressing GFP-DdRACK1 and GFP-RACK1mut after lysis by passing through Nucleopore filters. Protein aliquots separated by SDS PAGE were used to perform western blot analysis. WL, whole cell lysate; L, supernatant from cell lysate (400 x g); S1, P1 (10,000 x g); S2, P2 (100,000 x g). S, supernatant; P, pellet. DdRACK1 was detected with polyclonal anti-DdRACK1 antibodies. Antibodies against the cytosolic α-actinin were used as control.

A developmental analysis showed the presence of DdRACK1 protein in nearly unaltered levels during all stages of *Dictyostelium* development (Figure 11).

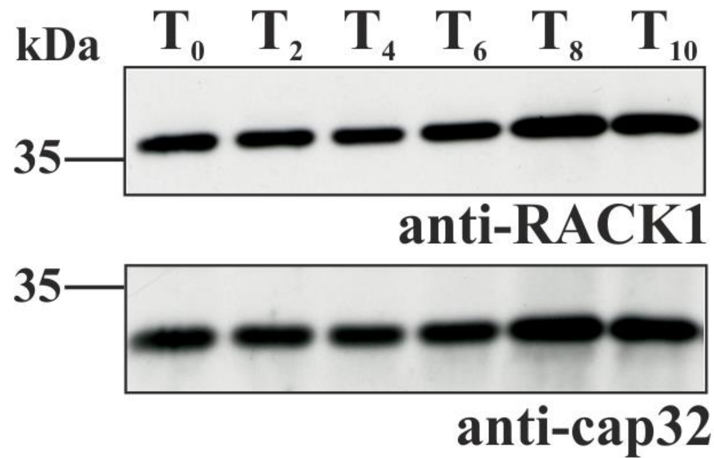


Figure 11 DdRACK expression levels during development. Western blot analysis was performed with AX2 wild type cell samples collected during starvation in shaking suspension at the indicated time points. DdRACK1 was detected with polyclonal anti-DdRACK1 antibodies. For loading control the blot was probed with mAb 188-19-95 detecting cap32.

3.4 DdRACK1 oligomerization potential

It has been reported that RACK1 can dimerize in vivo and this dimerization is required for specific processes including the regulation of the *N*-methyl-D-aspartate (NMDA) receptor by the Fyn kinase in the brain (Thornton *et al*., 2004). Here, we tested the capability of DdRACK1 to oligomerize using recombinant DdRACK1 full length protein that had been cleaved from the GST part. In the presence of the cross-linking reagent glutaraldehyde (0.001%), DdRACK1 formed dimers and even higher oligomers with increasing time of incubation as detected by western blots using polyclonal DdRACK1 specific antibodies. Interestingly, the native non-crosslinked DdRACK1 sample also contained some amount of dimers and oligomers (Figure 12). This indicates that the dimerization characteristic exhibited by RACK1 proteins also holds true for DdRACK1.

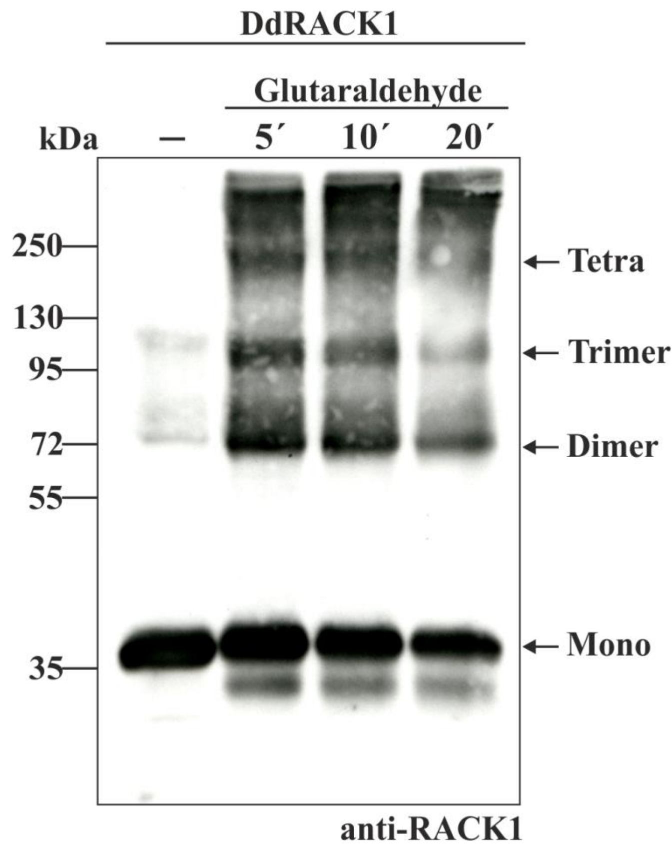


Figure 12 DdRACK1 forms homodimers and oligomers. 5-10 µg/100 µl of recombinant DdRACK1 were incubated with 0.001% of glutaraldehyde and samples taken at the indicated time points of 5, 10 and 20 min. In the absence of glutaraldehyde, the monomer (36 kDa, mono) and low amounts of dimer (72 kDa), and trimer (108 kDa) were detected. Proteins were detected with polyclonal anti-DdRACK1 antibodies. The band below the monomer is due to intramolecular crosslinks.

Similarly, DdRACK1mut in which we had exchanged the basic amino acid residues in the loop between blades 6 and 7 (see below, 3.6) also displayed wild type DdRACK1 oligomerization capability (Figure 13).

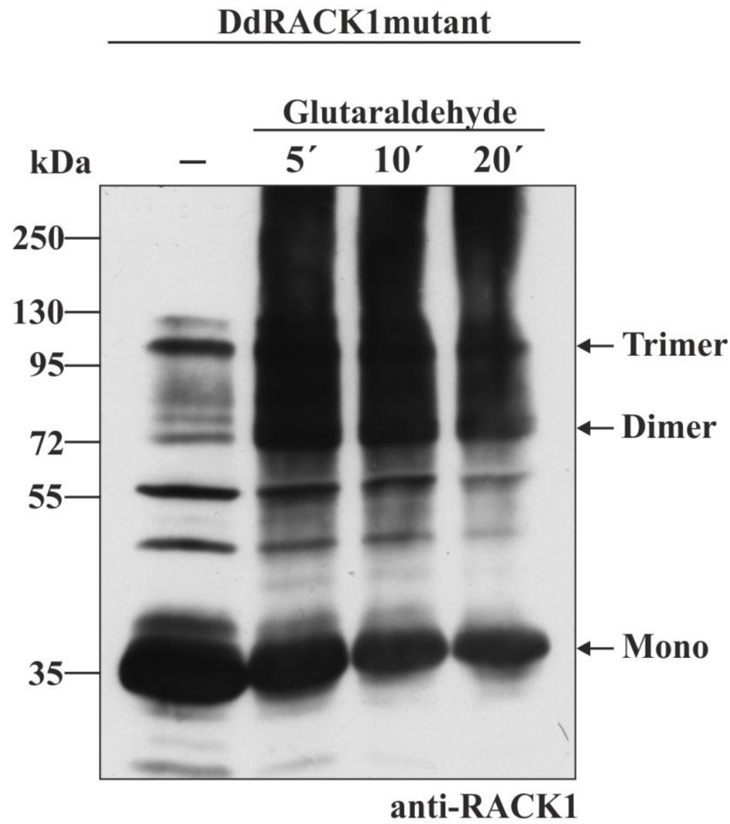


Figure 13 DdRACK1mut forms homodimers and oligomers. 5-10 µg/100 µl of recombinant DdRACK1mut were incubated with 0.001% of glutaraldehyde and samples taken at the indicated time points of 5, 10 and 20 min. For DdRACK1 and DdRACK1mut, in the absence of glutaraldehyde, the expexted monomers (36 kDa, mono), including dimers (72 kDa), and trimers (108 kDa) were detected. Proteins were detected with polyclonal anti-DdRACK1 antibodies.

We further confirmed DdRACK1 dimerization by co-immunoprecipitation assays. Both GFP-DdRACK1 and GFP-DdRACK1mut bound to GFP-trap beads precipitated endogenous DdRACK1 (Figure 14).

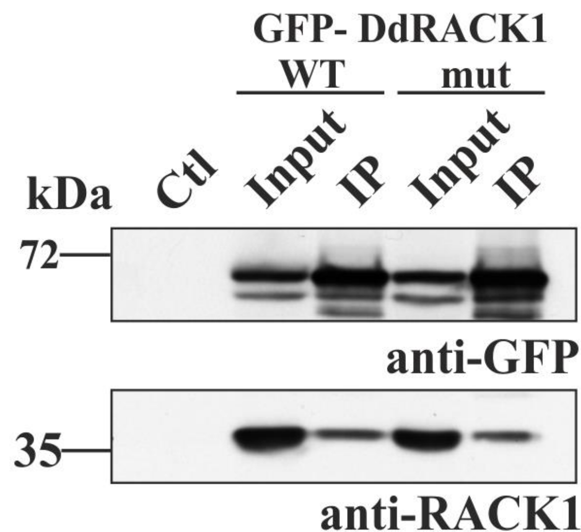


Figure 14 DdRACK1 forms homodimers and oligomers. Coimmunoprecipitation analysis to confirm DdRACK1 oligomerization. Both GFP-DdRACK1 and GFP-DdRACK1mut bound to GFP-trap beads (upper panel) were able to precipitate endogenous RACK1 (lower panel) from cells expressing GFP-tagged proteins. For GFP-RACK1 fusions a degradation band was observed. GFP-trap beads incubated with AX2 cell lysates were used as control (Ctl).

3.5 Post-translational modification of DdRACK1

Little is known about post-translational modifications of RACK1 apart from phosphorylation which is emerging as an important factor that modulates the binding of proteins to RACK1 (Adams *et al*., 2011). Phosphorylation of specific tyrosine residues and their corresponding functions have been reported. To determine if DdRACK1 also possesses the potential of becoming phosphorylated, we enriched DdRACK1 by immunoprecipitating GFP-DdRACK1 from cell lysates that were prepared in the presence or absence of phosphatase inhibitor cocktail (PIC) (Roche) and performed a western blot analysis using phosphotyrosine specific mAb 5E7 antibodies (Fendly *et al*., 1990). These antibodies recognized the GFP-DdRACK1

band on the blot indicating that DdRACK1, like RACK1 proteins from other species, can be phosphorylated on specific tyrosine residues (Figure 15).

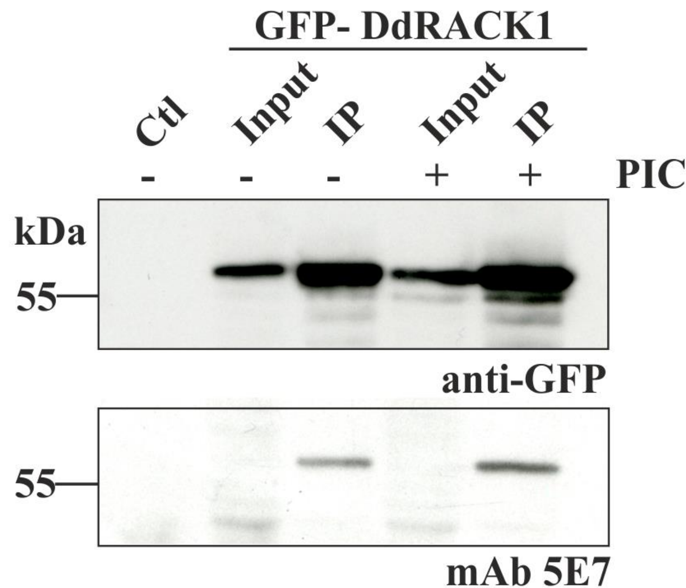


Figure 15 DdRACK1 is a phosphotyrosine-containing protein. Western blot analysis was performed with proteins from immunoprecipitated GFP-DdRACK1 cell lysates (upper panel) prepared in the presence or absence of phosphatase inhibitor cocktail (PIC). AX2 cell lysates incubated with GFP-trap beads was used as control (Ctl). The phosphotyrosine specific mAb 5E7 detected GFP-DdRACK1 in the IP (lower panel).

3.6 Lipid interactions

The mechanism of membrane association of DdRACK1 is not known. In general, membrane association of proteins can be achieved by various mechanisms. For instance, polybasic clusters as defined by arginine- and lysine-enriched amino acid sequences enable diverse transmembrane and cytosolic proteins to bind lipids (DeFord-Watts *et al.*, 2011). Also, proteins can target specific membranes through an interaction with phosphoinositides (PIPs). Based on the initial characterization of RACK1 as an interactor of RpkA we tested the ability of DdRACK1 to bind to different phosphoinositides in vitro using GST-DdRACK1 in dot-

blot (PIP strips) overlay assays. Whereas GST alone showed no PIP binding, GST-DdRACK1 bound with almost the same affinity to all the monophosphorylated PIPs, except for PI(3)P for which we observed stronger binding, to the bisphosphorylated PIPs as well as to the triphosphorylated PIP. GST-DdRACK1 also bound to phosphatidylserine (Figure 16). The *A. thaliana* and *D. discoideum* RACK1 proteins carry an insertion between propeller blades 6 and 7, which contains primarily basic amino acids, in case of DdRACK1 six lysine residues (Figures 3 and 5). By charge-reversal mutation, four lysine residues from the polybasic stretch were replaced with glutamic acid to generate a GST-fusion mutant version of DdRACK1 (GST-DdRACK1mut). The mutant protein when used in dot-blot protein overlay assays still bound to PI(4,5)P2 and PI(3,4,5)P3 although with reduced affinity, whereas binding to all other PIP variants was completely abolished (Figure 16).

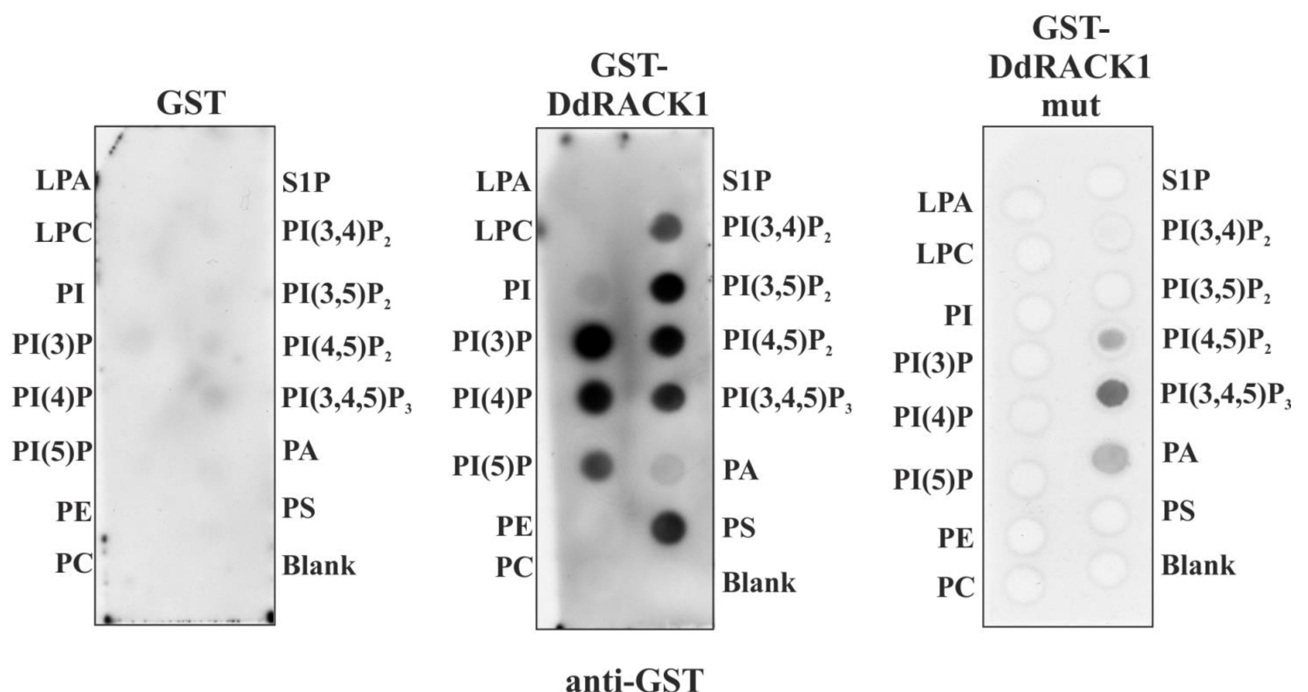


Figure 16 DdRACK1 binds to Phosphoinositides. PIP-Strip-membranes were incubated for 1 h at room temperature with 1 µg/ml GST (control), GST-DdRACK1 and GST-

DdRACK1mut respectively. Binding to PIPs on membranes was detected by incubation with polyclonal anti-GST antibodies.

Although dot-blot overlay assays are convenient assays, they need to be supported by different methods as apparent specificities may be distorted and as they do not allow reliable quantification (Narayan and Lemmon, 2006). We therefore examined the sedimentation of GST-DdRACK1 with liposomes containing 65% phosphatidylcholine, 20% phosphatidylethanolamine, 5% phosphatidylserine, reconstituted with 10% individual phosphoinositides. Although without any specificity, while GST-DdRACK1 showed significant binding to these liposomes, indicating a broad binding specificity for membranes, GST-RACK1mut did not show significant binding to any of the PIPs. This supports the requirement of this polybasic region by RACK1 for lipid binding. GST was included as a control and did not sediment with the liposomes (Figure 17).

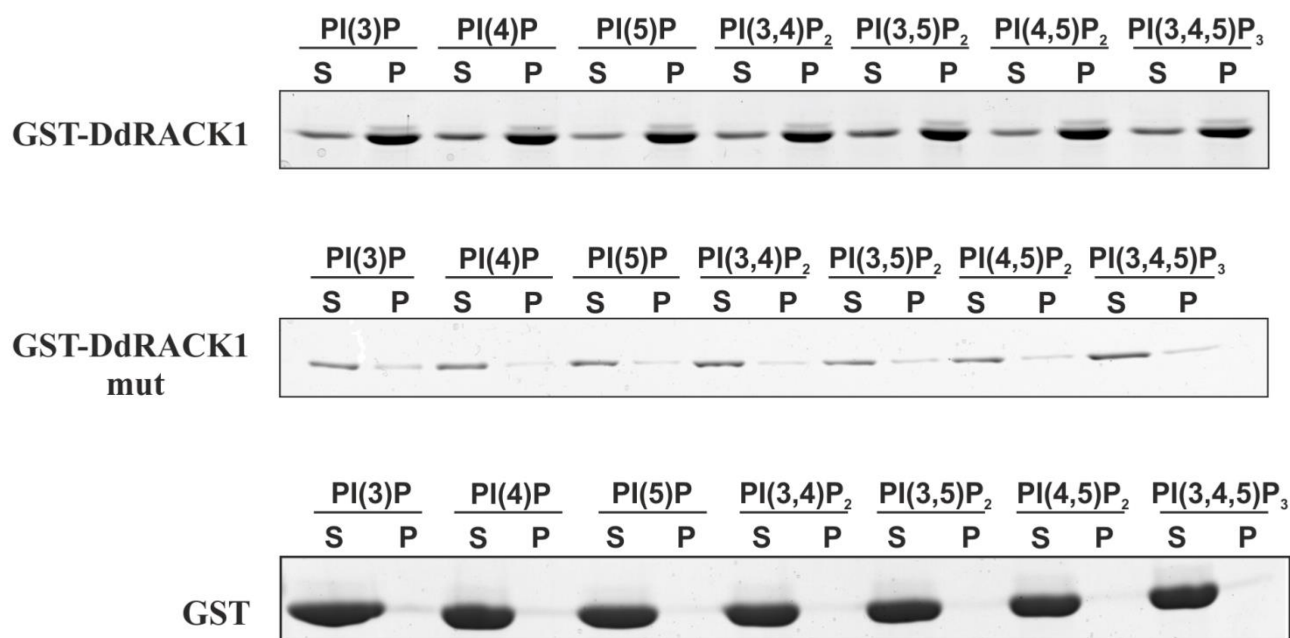


Figure 17 Binding of GST-DdRACK1, GST-DdRACK1mut and GST (control) to PIPs in a liposome binding assay. 5-10 µg of GST and the GST-fusion proteins were

incubated with liposomes containing 10% (wt/wt) of the indicated PIPs. Liposomes were collected by centrifugation, and bound proteins resolved by SDS-PAGE and detected by Coomassie Blue staining (S = supernatant; P = pellet).

To quantitatively study to which PIPs DdRACK1 preferable bound, band intensities of the Coomassie Blue stained gels from both DdRACK1 and DdRACK1mut binding assays were scanned and the pellet fractions plotted. This assay showed that DdRACK1 interacted equally well with all the different PIPs. Generally, while more than 60% of DdRACK1 bound to the PIPs liposomes, only less than 20% DdRACK1mut were bound (Figure 18).

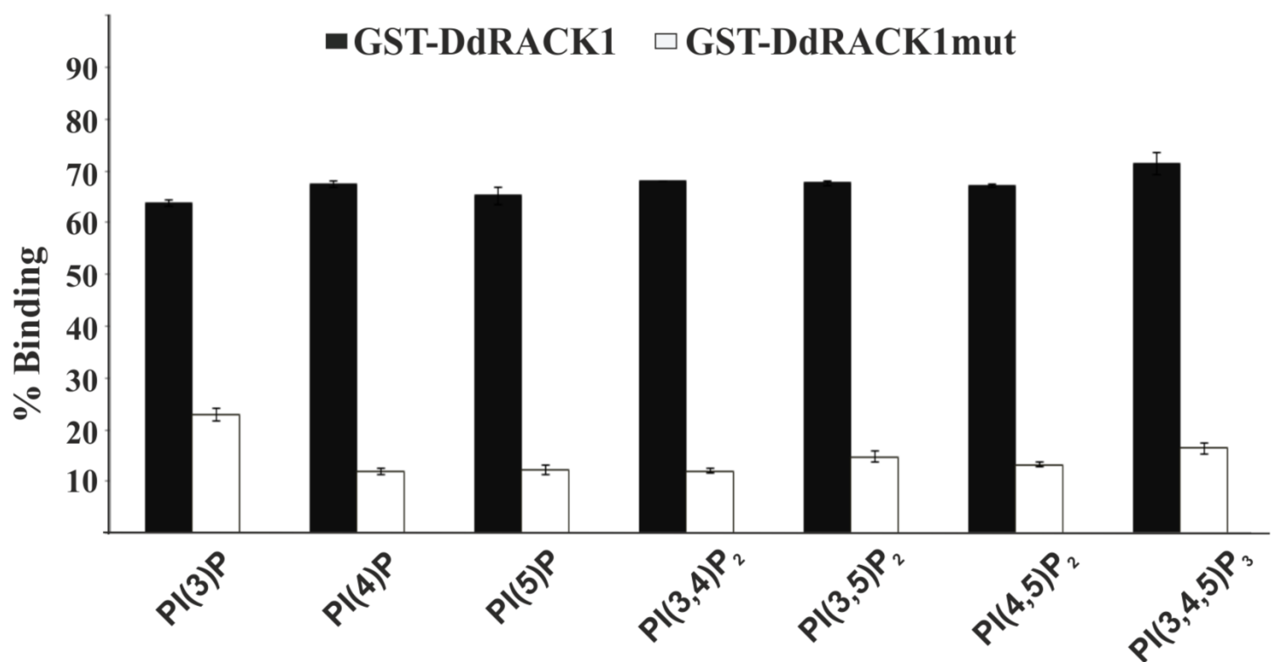


Figure 18 Quantification of bound GST-DdARCK1 and GST-DdRACK1mut in pellet samples from Figure 17. Protein bands were quantified with ImageJ software.

3.7 DdRACK1 interacts with G proteins

Conventional Gβ subunits exhibit a high affinity for Gγ subunits and function as Gβγ heterodimers to bind and stabilize GDP-bound Gα subunits. In addition, a Gβ can associate

with multiple individual Gγ subunits (Clapham and Neer, 1997). The interaction of Gβγ with RACK1 was first identified by a yeast two-hybrid screen using the bovine Gβ1 sequence as bait to screen a mouse brain library (Dell *et al*., 2002). To test whether DdRACK1 likewise associates with the *D. discoideum* Gβ and Gγ protein subunits, we performed a yeast two-hybrid analysis using DdRACK1 fused to the pACT2-AD. Gβ and Gγ subunits were fused to pAS2-BD, respectively. We detected interactions between DdRACK1 and Gβ as well as between DdRACK1 and Gγ subunits as revealed by β-galactosidase production (Figure 19, blue staining of the colonies). For *S. cerevisiae* it is reported that the RACK1 orthologue Asc1p functions as a Gβ subunit for a Gα (Gpa2) (Zeller *et al*., 2007). Similarly, in the human pathogenic fungus *C. neoformans* the RACK1 orthologue Gib2 functions as Gβ for Gpa1 (Palmer *et al*., 2006). Like *D. discoideum* both organisms have a single Gβ gene. To investigate DdRACK1-Gα interactions, yeast two-hybrid assays were performed using DdRACK1 fused to pACT2-AD. The Gα subunits Gα1, Gα2, Gα4, Gα5, Gα6, Gα7, Gα8, Gα9, Gα10, Gα11 and Gα12 were fused to pAS2-BD. In these assays we detected interactions between DdRACK1 and Gα2 as well as between DdRACK1 and Gα8 (Figure 19 and data not shown).

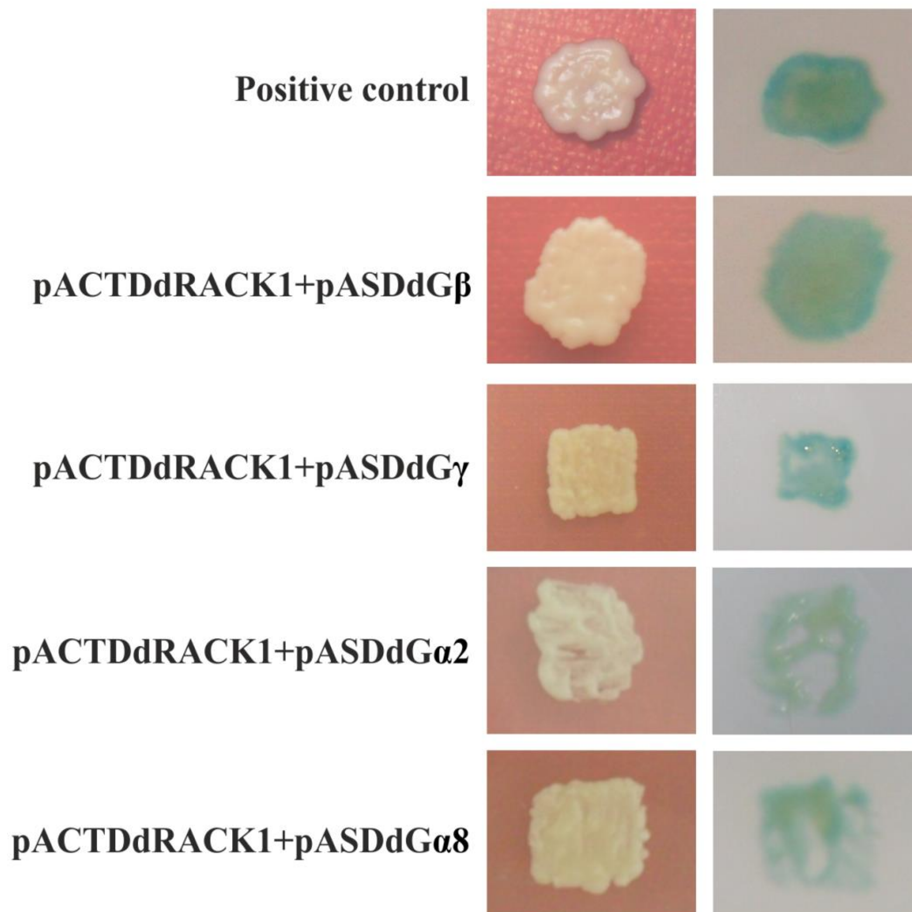


Figure 19 Yeast two-hybrid analyses and β-galactosidase activity staining. Yeast Y190 strain that has *lacZ* and *His3* reporter genes was co-transformed with DdRACK1 in pACT2 vector and the Gβ, Gγ, Gα2 and Gα8 protein subunits in pAS2 vector, respectively. β-galactosidase activity staining in X-gal solution was then performed with selected colonies from Pre-SD agar plates.

To confirm these interactions we performed co-immunoprecipitation analyses. GFP-Gβ, Gγ-YFP, Gα2-RFP, Gα4-RFP and Gα8-RFP expressed in AX2 and bound to GFP- and RFP-trap beads respectively co-immunoprecipitated endogenous DdRACK1 (Figure 20). This provides further evidence that DdRACK1 resembles RACK1 proteins and, like those, interacts with G proteins.

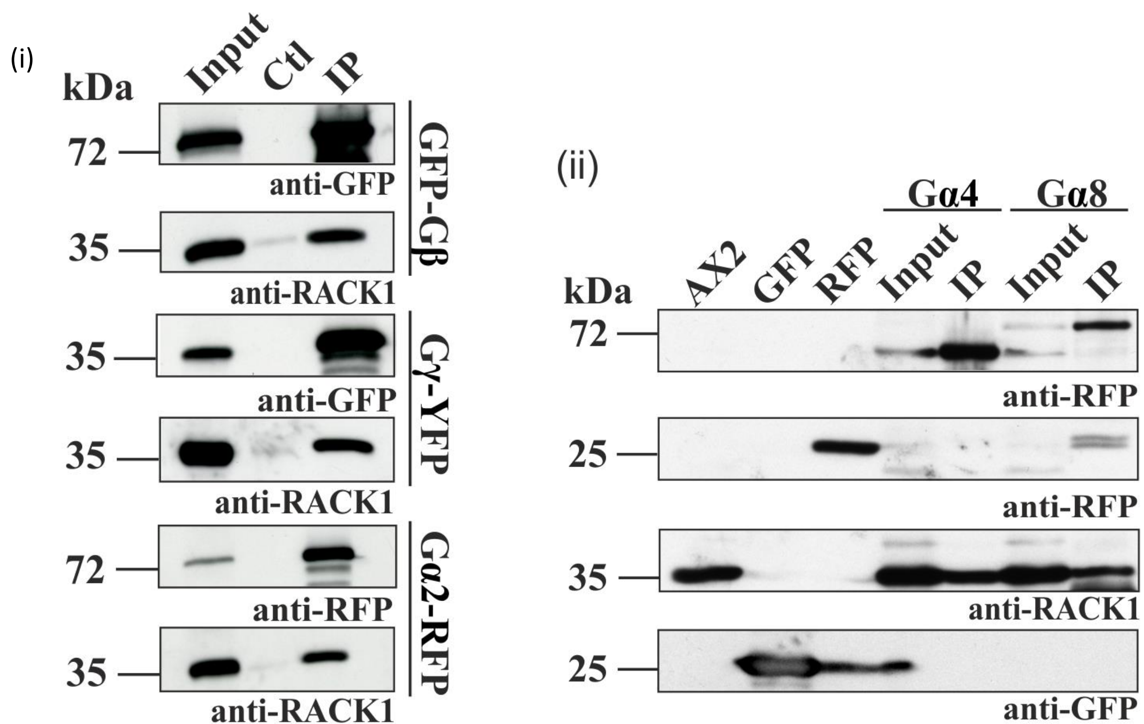


Figure 20 Co-immunoprecipitation assays to confirm DdRACK1 interaction with the Gβ, Gγ and Gα2 protein subunits. (i) GFP-Gβ, Gγ-YFP bound to GFP-trap beads and Gα2-RFP bound to RFP-trap beads co-precipitated endogenous DdRACK1 (IP). GFP-trap beads incubated with AX2 cell lysates were used for control (Ctl). (ii) Gα4 and Gα8 bound to RFP-trap beads co-precipitated endogenous DdRACK1 (IP). GFP and RFP bound to beads were used as control. mAb K3-184-2 detected GFP-Gβ and Gγ-YFP; mAb K73-875-7 detected Gα2-RFP, Gα4-RFP and Gα8-RFP. DdRACK1 was detected with polyclonal anti-DdRACK1 antibodies.

The interactions were further confirmed with pulldown experiments. GST-Gα2, GST-Gα4, GST-Gα8 as well as GST-Gγ pulled down endogenous DdRACK1 (Figure 21).

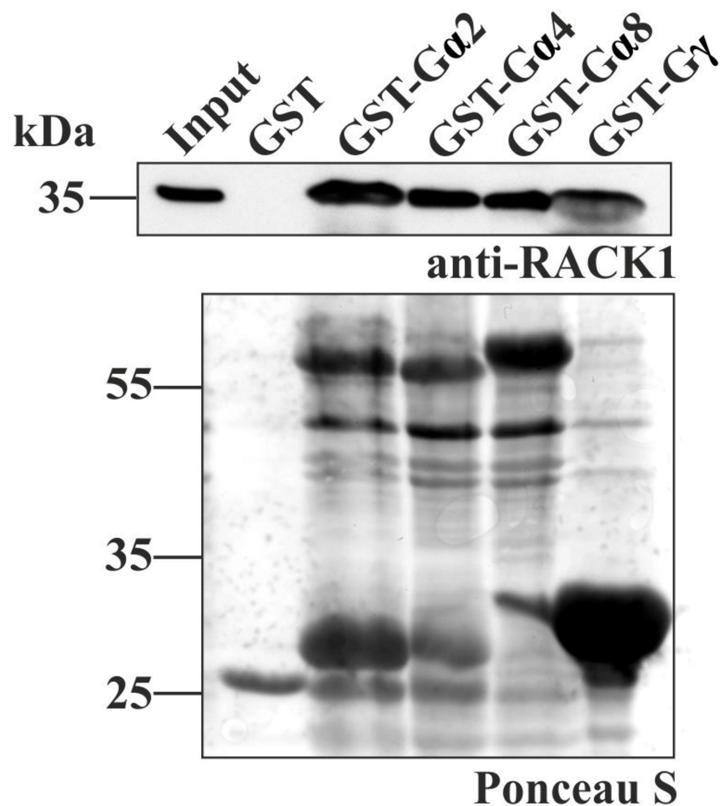


Figure 21 GST pulldown experiments to confirm DdRACK1 interaction with Gα subunits 2, 4 and 8, and γ respectively. DdRACK1 in the input and in the precipitates was detected with polyclonal anti-DdRACK1 antibodies. Below the proteins employed in the pulldown are shown by staining of the nitrocellulose membrane with Ponceau S.

To analyse if DdRACK1 also takes over the Gβ function for the Gα subunits in vivo we ectopically expressed DdRACK1 as a GFP fusion in the *gβ* null mutant LW6 (Wu *et al.*, 1995; Peracino *et al.*, 1998) and analysed whether it rescues the impaired growth on a bacterial lawn, the phagocytosis, chemotaxis, aggregation and developmental defects. We found that expression of GFP-DdRACK1 in LW6 cells did not rescue the developmental defect. When we plated the cells on a lawn of *K. aerogenes* they formed smooth plaques as observed for the mutant strain (Figure 22).

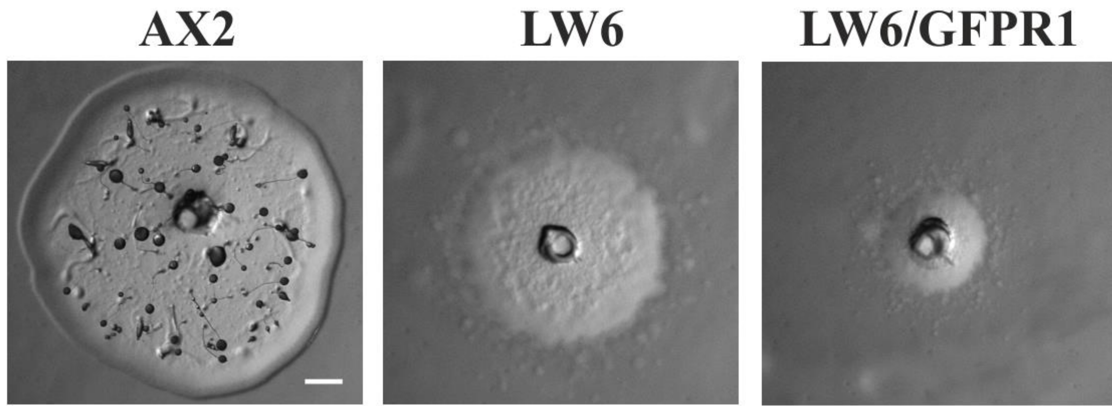


Figure 22 Growth and development of AX2, *gβ* null mutants (LW6) and LW6/GFP-DdRACK1 cells plated on a *K. aerogenes* lawn. Images taken after 3 days are shown. Scale bar, 0.5 mm.

Remarkably, the plaque size was even further reduced when we compared the wild-type strain AX2, LW6 and LW6 expressing GFP-RACK1 (Figure 23). This might be due to a further reduction in the rate of phagocytosis or enhanced defects in cell motility.

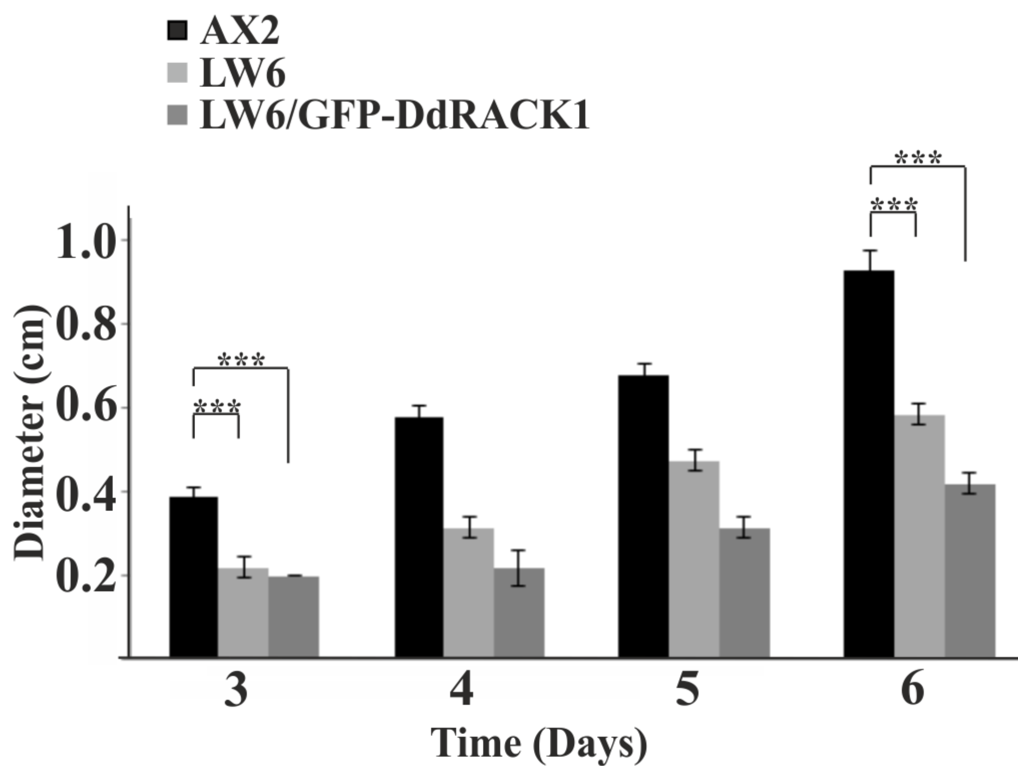


Figure 23 Measurement of plaque diameter to determine plaque size formed by AX2, LW6 and LW6/GFP-DdRACK1 cells over several days. The bar represents the mean and SD of ten independent experiments (****P* < 0.001).**

Hence we examined their phagocytic capability following yeast particle uptake and found that whereas fewer LW6 cells had ingested one or more yeast particles after 30 min as expected when compared with AX2 cells, even fewer LW6/GFP-DdRACK1 cells took up yeast cells. Quantitatively, ~24% LW6 and ~15% LW6 cells expressing GFP-DdRACK1 had taken up yeast cells as compared to ~64% uptake level for AX2 (Figure 24).

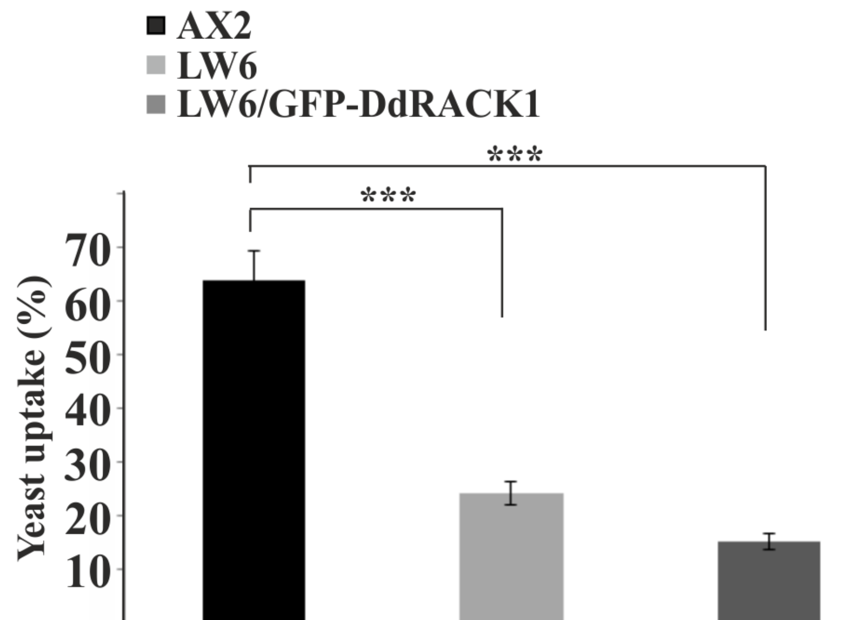


Figure 24 Yeast uptake experiments were performed using the strains from Figure 22 and TRITC-labelled yeast to determine their phagocytic capabilities. Approximately 200 cells from each strain were counted. The percentage of cells which had engulfed yeast after 30 min is shown on the graph (****P* < 0.001).**

3.8 Growth and development of *D. discoideum* strains

Our attempts to generate *D. discoideum* knockout mutants for RACK1 were not successful. Since RACK1 acts as a scaffold protein, interference with its levels might lead to cellular defects which can give an indication about its involvement in critical cellular roles. We therefore tried to study the effects of RACK1 overexpression in a wild type background and characterized AX2 cells expressing GFP-DdRACK1 and GFP-DdRACK1mut. From western blot analysis with AX2, AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut cells, we found that the levels of RACK1, with respect to endogenous RACK1, were only moderately enhanced (~17% in AX2/GFP-DdRACK1 and ~13% in AX2/GFP-DdRACK1mut cells respectively) (Figure 25). Such behaviour may be the result of the scaffolding function. It has been proposed that the levels of scaffold proteins should be tightly regulated as misregulation might interfere with many cellular processes (Ron *et al.*, 2013).

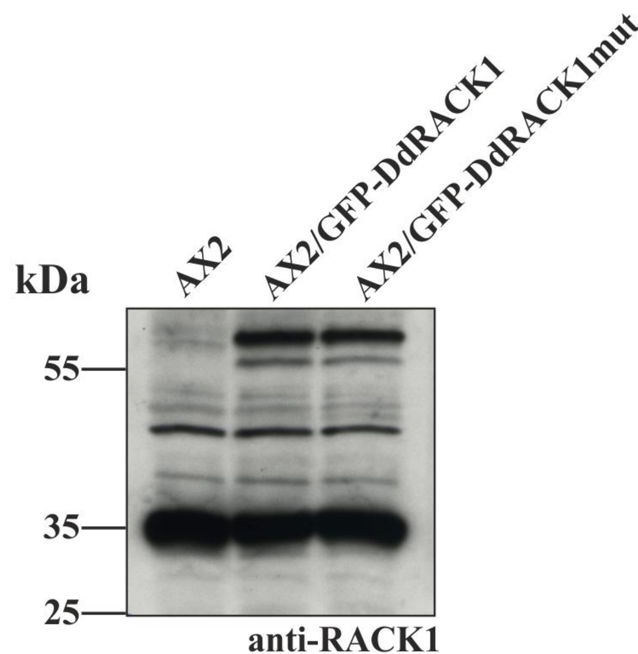


Figure 25 Levels of DdRACK1 overexpression. Cell lysates from vegetative AX2, AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut strains were analyzed by SDS-PAGE and western blot. DdRACK1 and GFP-fusion proteins at 36 and 66 kDa were detected with polyclonal anti-DdRACK1 antibodies.

Growth in shaking suspension was comparable between AX2 and AX2 expressing GFP-DdRACK1 and GFP-DdRACK1mut with similar duplication times and similar final densities (~$1x10^7$cells/ml). However, once the cells had reached maximum density, AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut cells did not stay in the stationary phase like AX2 as cell counts dropped rapidly (Figure 26).

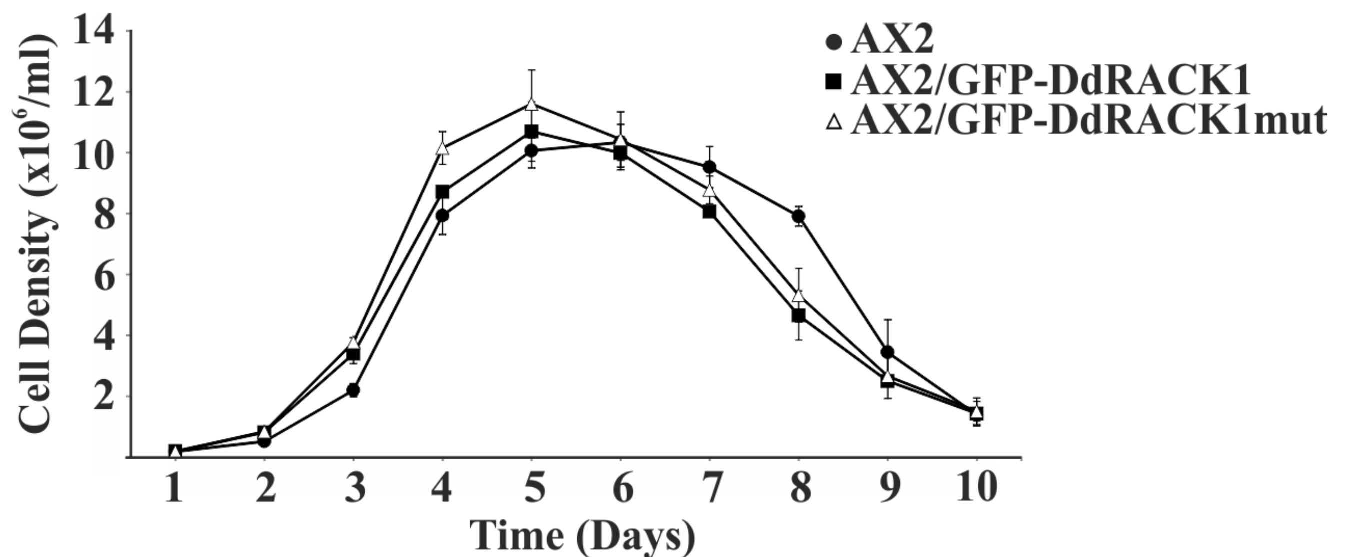


Figure 26 Growth in shaking suspension of *D. discoideum* strains as in Figure 25. $5x10^4$cells/ml was used for inoculation.

Differences were also observed during growth on lawns of *K. aerogenes* on SM agar and *E. coli* B12 on nutrient agar (NA) plates. In these assays we noticed an expanded region containing GFP-DdRACK1/AX2 cells when they were grown on *K. aerogenes*. Upon growth on a lawn of *E. coli* B12 this behaviour was even more conspicuous (Figure 27, 29).

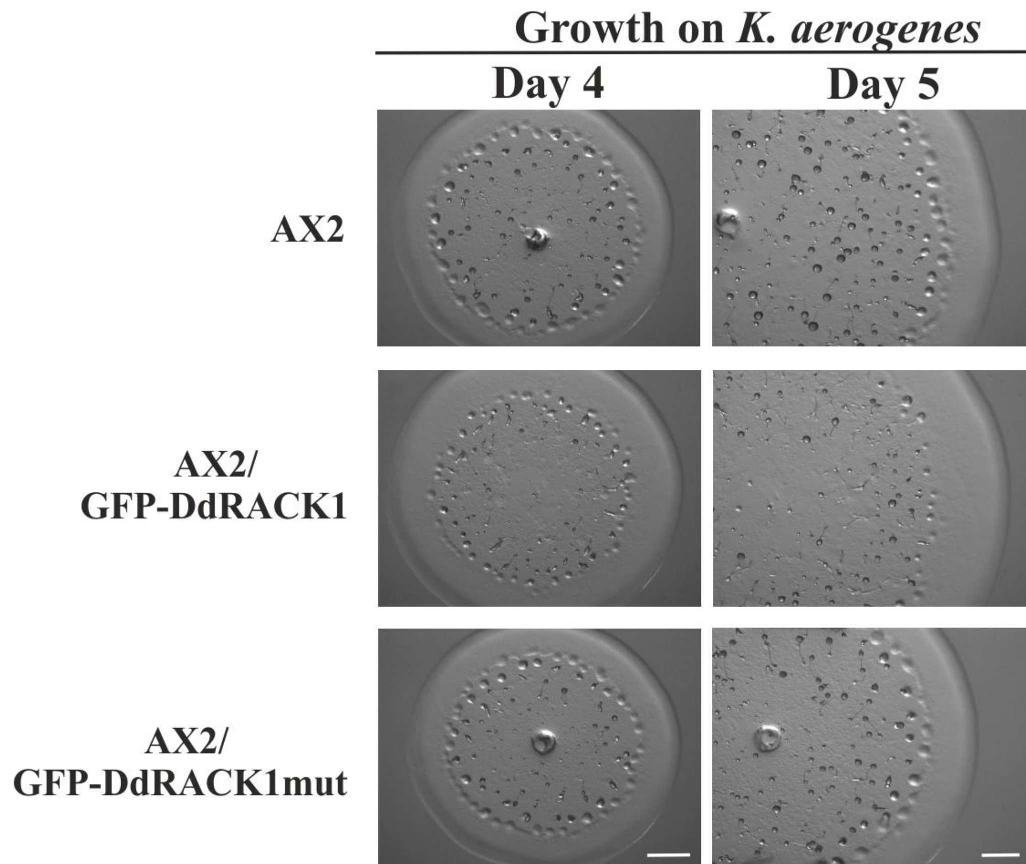


Figure 27 Growth on a lawn of *K. aerogenes* of *D. discoideum* strains as in Figure 25. Images were taken on days 4 and 5. Scale bar, 1 mm.

AX2 cells expressing GFP-DdRACK1mut were like wild type (Figure 27). AX2, AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut displayed similar growth rate on a lawn of *K. aerogenes* when plaque diameter was measured (Figure 28).

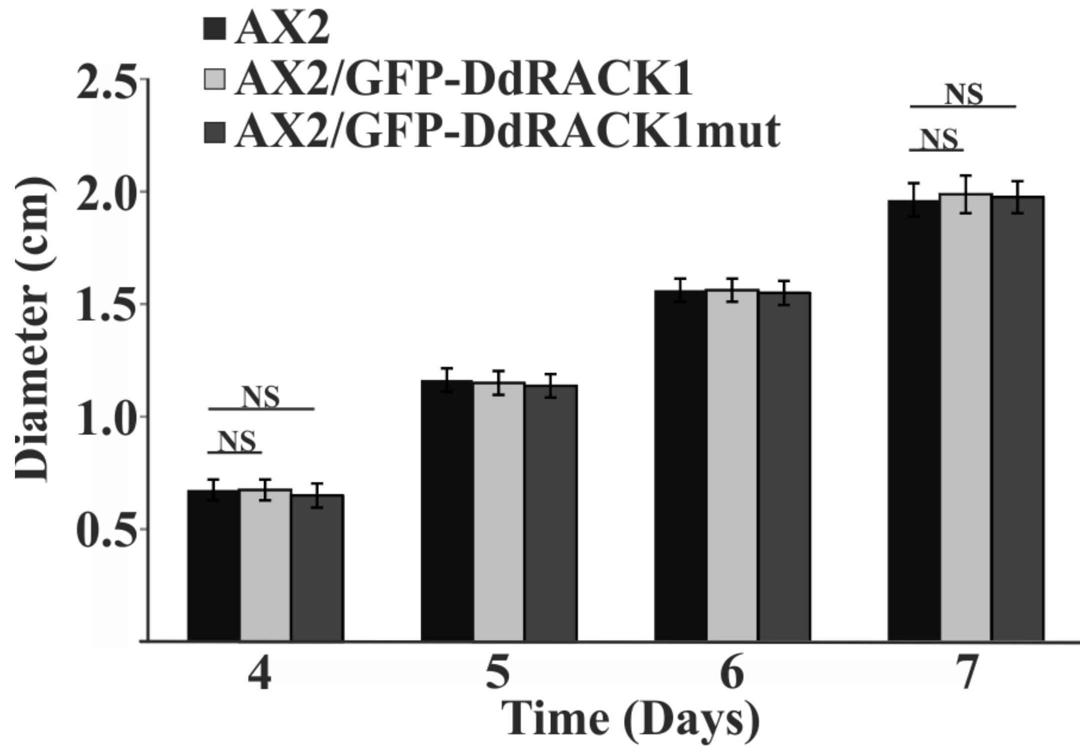


Figure 28 Bar chart showing diameter of plaques formed by *D. discoideum* strains in Figure 27 measured between days 4 and 7. The bar represents the mean and SD of ten independent experiments (NS, not significant; $P > 0.05$).

On an *E. coli* lawn the AX2/GFP-DdRACK1 strain showed increased growth whereas AX2 and AX2/GFP-DdRACK1mut displayed similar growth behaviour (Figure 29 and 30). Faster growth on a bacterial lawn could be due to increased phagocytosis, altered cell motility or to a developmental defect.

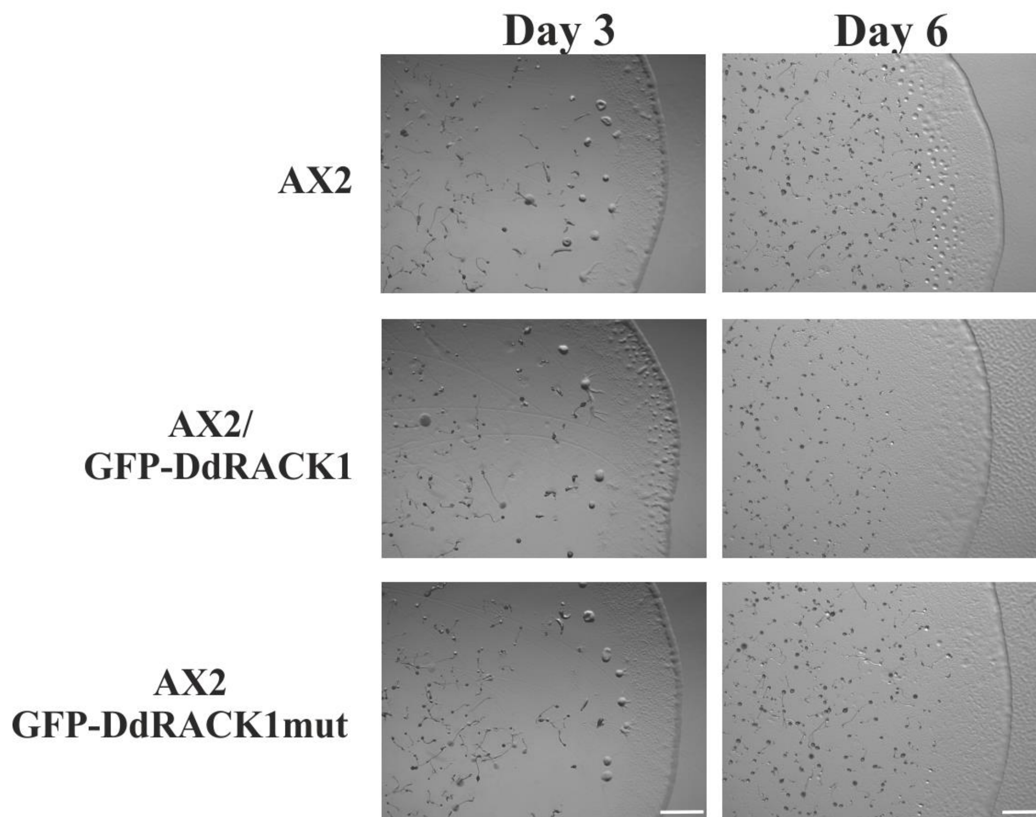


Figure 29 Growth of *D. discoideum* strains as in Figure 25 on a lawn of *E. coli* B12 as imaged on days 3 and 6. Scale bar, 1 mm.

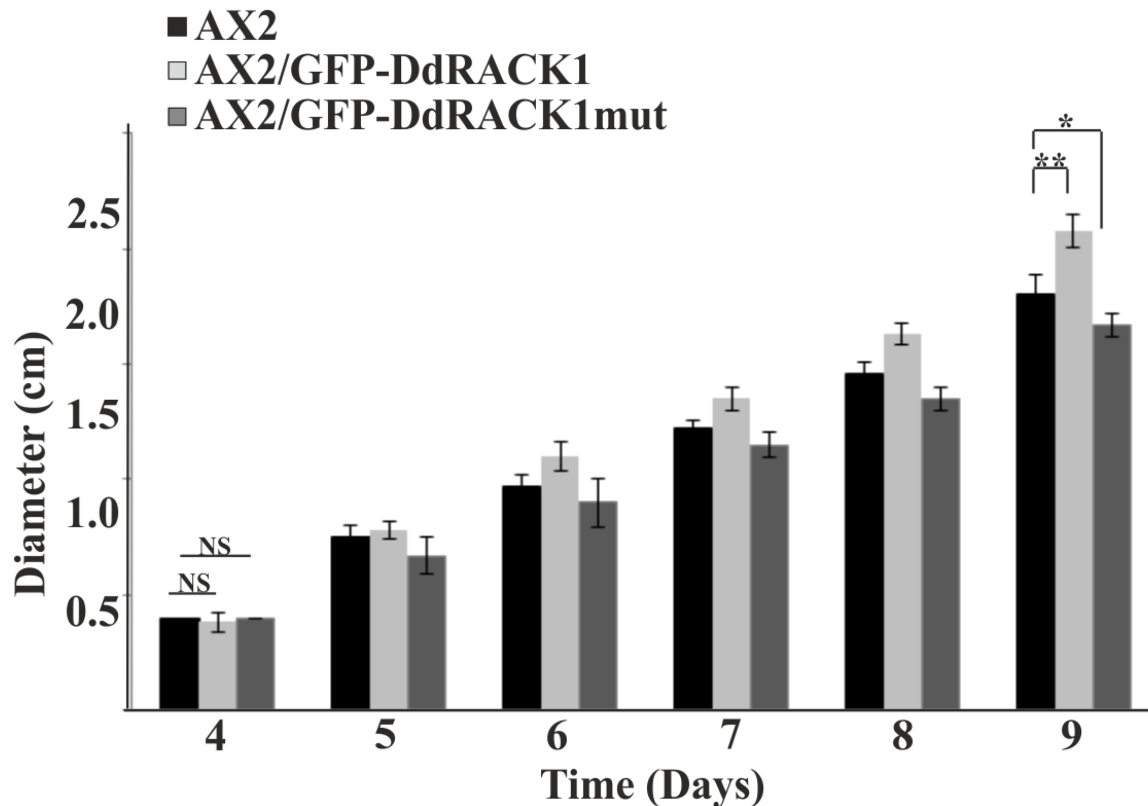


Figure 30 Bar chart showing diameter of plaques formed by *D. discoideum* strains in Figure 29 measured between days 4 and 9. The bar represents the mean and SD of ten independent experiments (*P* < 0.01; **P* < 0.05; NS, not significant; *P* > 0.05).**

3.9 Development is altered in DdRACK1 overexpressor strains

D. discoideum development is initiated by starvation. AX2 cells plated on phosphate agar plates start to form multicellular aggregates between 8 to 12 hours and have formed fully differentiated fruiting bodies after ~24 hours. In our experiments cells from all strains had gathered into mounds at 10 hours. After 24 hours AX2 cells and AX2 expressing GFP (AX2/GFP) had formed fruiting bodies, whereas those of both AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut were still present as tight aggregates and fruiting bodies were observed only after 42 hours. They were smaller than those of AX2 and there were still many mounds present (Figure 31).

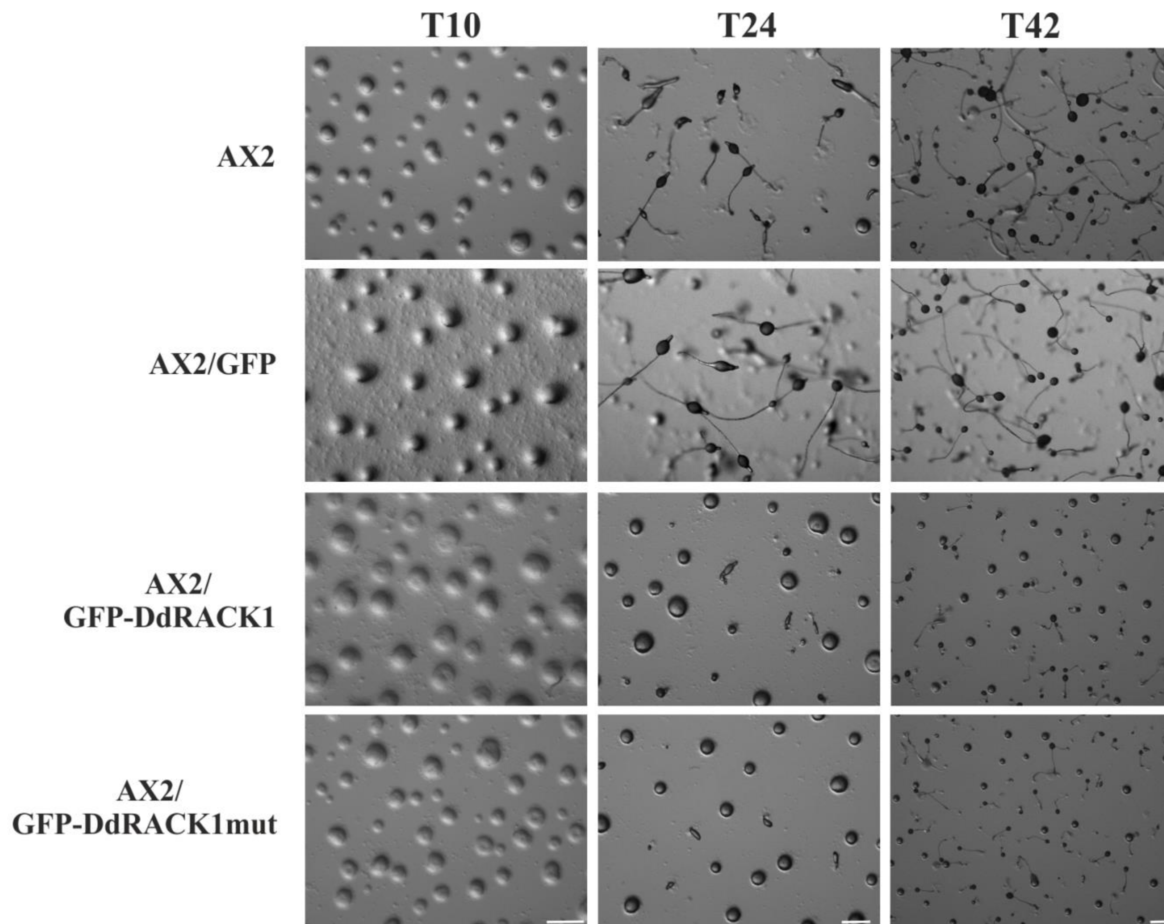


Figure 31 5 x 10^7 AX2, AX2/GFP, AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut cells were deposited on phosphate agar plates and imaged at the indicated hours of development. Scale bar, 250 µm.

To investigate development further, we examined the aggregation behaviours of these strains on a plastic surface. AX2 cells were highly elongated and formed well-defined streams after 9 hours of starvation. After 11 hours the streams became thicker and shorter. AX2/GFP-DdRACK1 and AX2/GFP-DdRACK1mut cells failed to form streams after 9 hours. AX2/GFP-DdRACK1 cells started to form aggregates after 11 hours, start of aggregate formation was even more delayed in AX2/GFP-DdRACK1mut cells (Figure 32).

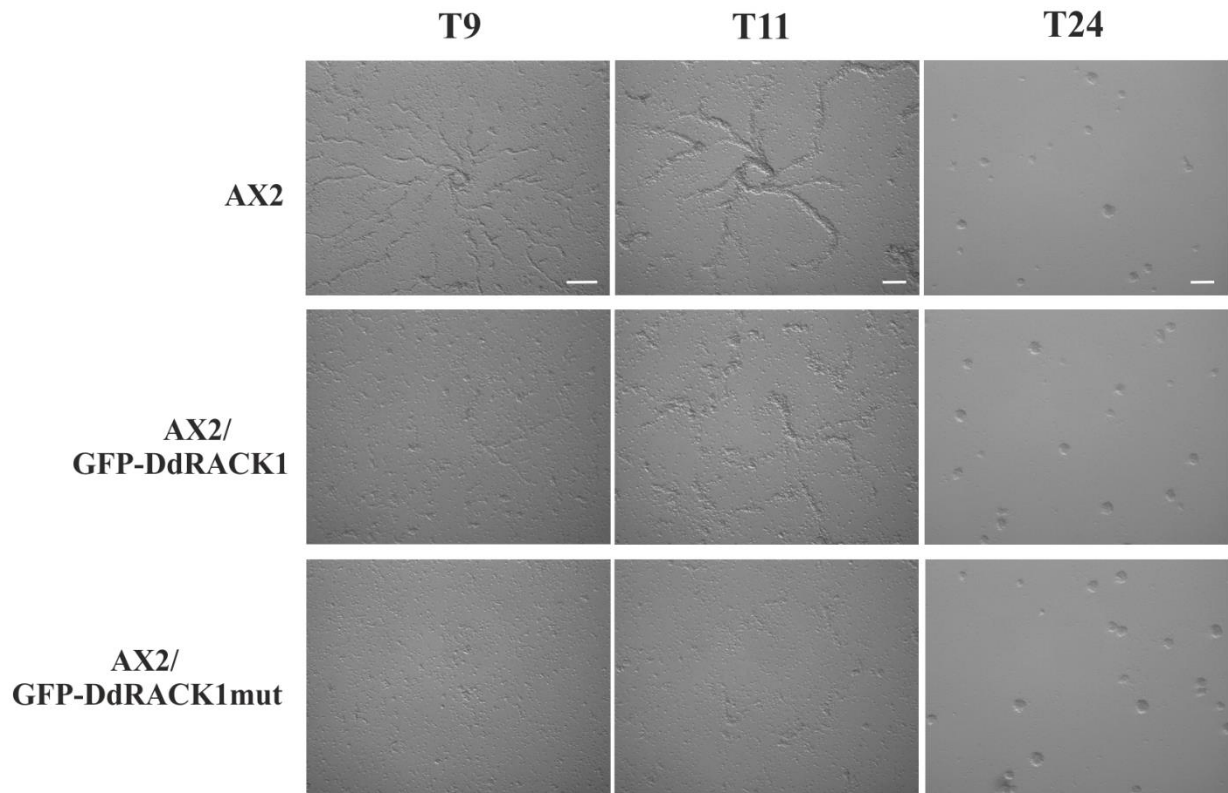


Figure 32 1 x 10^7 *D. discoideum* cells of the different strains were starved on petri dishes under phosphate buffer. Images were taken at the indicated time points. Scale bar, 250 µm.

When we monitored the expression of the strictly developmentally regulated cell adhesion protein contact site A (csA) in cells starved in shaking suspension, we observed a similar expression pattern with a first detection after four hours of starvation and a steady increase in all three strain (Figure 33).

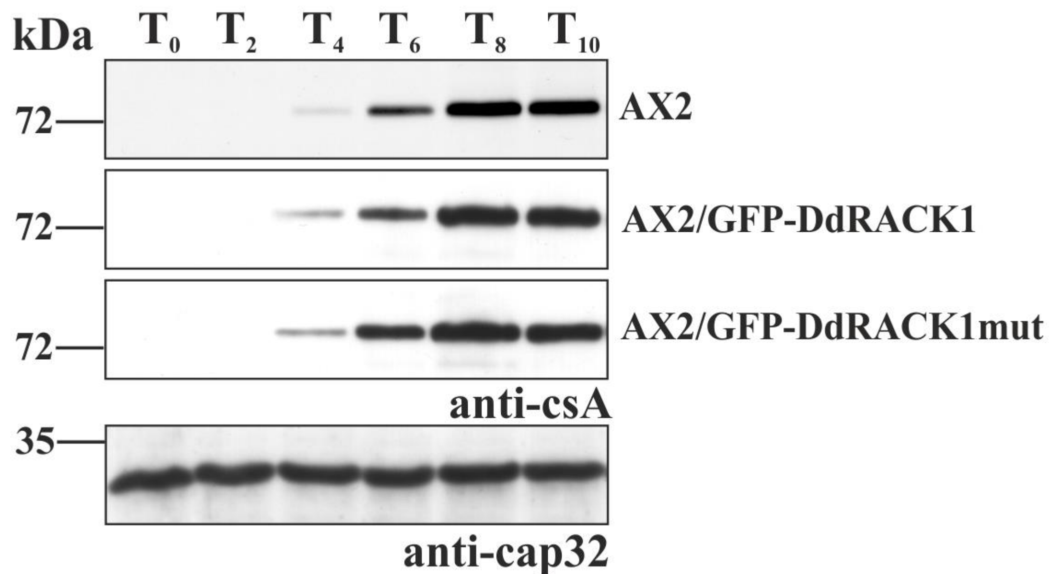


Figure 33 Time-dependent expression of csA. Cells from the different *D. discoideum* strains were collected during development in shaking suspension at the indicated time points and analyzed by SDS-PAGE and western blots. csA was detected by mAb 33-294, mAb 188-19-95 detected cap32 which was used as loading control.

This was however not the case when we monitored csA expression from cells starved on plates under phosphate buffer. AX2 and AX2/GFP-DdRACK1 showed similar expression pattern with a first detection of csA after six hours of starvation, AX2/GFP-DdRACK1mut cells showed delayed csA expression with first detection after eight hours of starvation (Figure 34). This corroborates the data obtained by visual inspection to a certain extent (Figure 32).

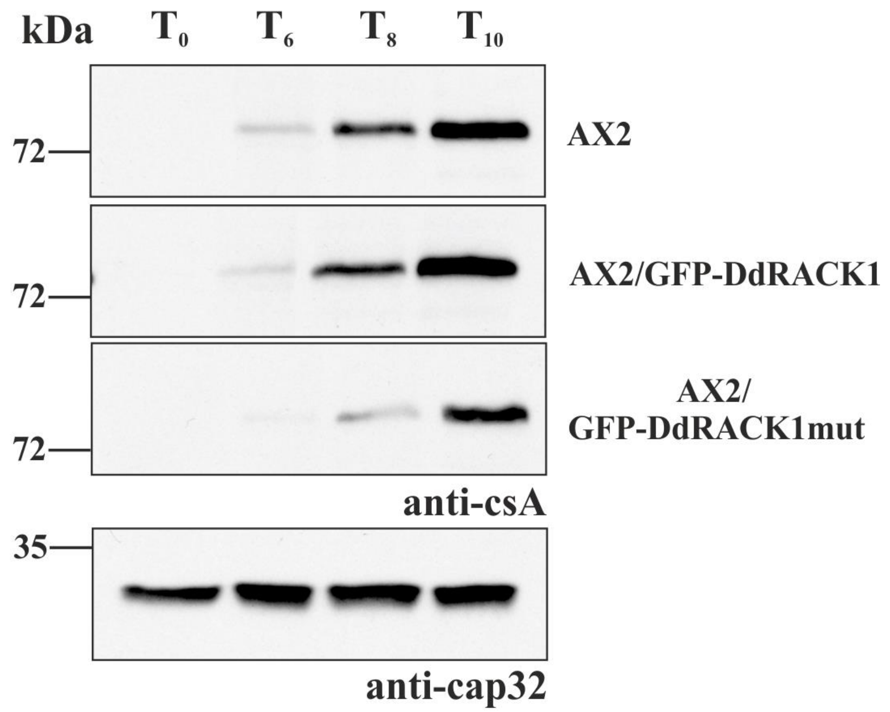


Figure 34 Cell samples of *D. discoideum* strains collected from submerged cultures in phosphate buffer were tested for csA expression.

4 Discussion

Scaffold proteins uniquely integrate signals from multiple pathways. They generate lots of functional diversity by mediating a series of interactions with a vast array of protein partners. The receptor for activated C kinase 1 (RACK1) is a member of the evolutionarily conserved family of WD40 repeat proteins which forms seven β-propeller blades. It was initially discovered through its ability to function as a scaffold protein, bringing in close proximity protein kinase C (PKC) and its substrates (Ron and Mochly-Rosen, 1995; Ron *et al*., 1994). In this study we report a novel protein in *D. discoideum* that is hitherto uncharacterized and displays very significant identity to RACK1 proteins that have been very well studied in various other species. Due to its high similarity to these other RACK1 proteins we have named this protein DdRACK1.

DdRACK1 is a WD40 repeat protein harboring a seven-bladed β-propeller that shares some similarities with the heterotrimeric G protein β subunit. The modelled structure of DdRACK1 features the seven β-propeller architecture with each propeller blade arranged in sequential order and made up of four-stranded antiparallel β-sheets. Although differences exist, particularly in the extended loop that connects β-propeller blades 6 and 7, the structures of RACK1 from *S. cerevisiae* (Yatime *et al*., 2011), *A. thaliana* (Ullah *et al*., 2008) and human (Ruiz *et al*., 2012) show significant identity with DdRACK1. The region between the β-propeller blades 6 and 7 is conserved between DdRACK1 and *A. thaliana* RACK1A. The major difference between the WD repeats is in the loops that provide the distinct features of each member of the WD family and distinguishes RACK1 interactions from those of other WD proteins (Garcia-Higuera *et al*., 1996; Sklan *et al*., 2006). The *A. thaliana* protein was the first RACK1 orthologue to be structurally described (Adams *et al*., 2011). Unlike in *A. thaliana* where RACK1 is expressed by three genes, DdRACK1 is expressed like in

metazoans by only gene, *gpbB*. Residues in two conserved regions of RACK1 have been mapped in *A. thaliana* RACK1A (Ullah *et al*., 2008). These regions represent potential protein-protein interaction sites. The first conserved region is located on the top rim of the propeller and involves side chains from residues R36, K38, S63, H64 (blade 1), R42, K44, S70, H71; W83, D107 (blade 2), W90, D114; R125 (blade 3), R132; and W152 (blade 4), W158 in DdRACK1. The second large conserved surface region of RACK1 is located on the bottom of the propeller and is comprised of conserved residues P204, D205, Y230 (blade 5), P208, D209, Y234; and N246, Y248 and W249 (blade 6), N250, Y252 and W253 in DdRACK1. Besides the high sequence identity between DdRACK1 and RACK1 from other species, the presence and conservation of these above mentioned regions indicates that DdRACK1 is a member of the RACK1 family of WD40 repeats proteins and may undergo similar interactions. Although DdRACK1 is mainly cytosolic as seen from live confocal microscopy images and fractionation studies, a portion of it was also found in the membrane fraction, buttressing localization to cellular membranes by proteins of the RACK1 family. Furthermore, RFP- and GFP-DdRACK1 were found in *D. discoideum* membrane protrusions as well as in filopod-like extensions. Mutations in the basic loop of DdRACK1 did not alter its localization.

RACK1 dimerizes both in vivo and in vitro (Yatime *et al*., 2011; Thornton *et al*., 2004; Ruiz *et al*., 2012). The physiological role is however still unclear. In the regulation process of the NMDA receptor by Fyn, RACK1 dimerization is required to bring the two interacting partners in close contact. RACK1 dimerization allows exposing a new surface of the protein, buried within the propeller core in the monomeric form (Yatime *et al*., 2011). We have provided evidence that DdRACK1 also has the potential to dimerize. The dimerization of human RACK1 is enhanced by phosphorylation (Liu *et al*., 2007) and one of the putative

phosphorylation sites was Ser146 in blade 3. This residue is however not conserved in DdRACK1, but there are other Ser/Thr residues present in this region which could probably be potential targets in mediating DdRACK1 dimerization by phosphorylation. On the other hand, an important factor which modulates the binding of RACK1 proteins to partners is tyrosine phosphorylation (Adams *et al.*, 2011). Phosphorylation/dephosphorylation of different tyrosine residues of human RACK1 regulates various cellular processes (Kiely *et al.*, 2006; 2008, Chang *et al.*, 2002). These tyrosine residues are also conserved in DdRACK1, and we provided evidence that the DdRACK1 protein is a phosphotyrosine-containing protein.

Phosphoinositides (PIPs) regulate fundamental biological processes including cell growth and survival, membrane trafficking and cytoskeletal dynamics (Kutateladze, 2010). PIPs are tightly regulated during chemotaxis in *D. discoideum*, in particular, PI(3,4,5) P3 gradients are formed within the plasma membrane (Müller *et al.*, 2013). They are thought to be of differing importance for sensing of shallow and steep gradients (Kölsch *et al.*, 2008; Hoeller and Kay 2007). In the region between β-propeller blades 6 and 7 we noted a key polybasic cluster (-KKKK-) in DdRACK1 which turned out to be responsible for binding to several PIPs; PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3 without particular preference; and also to phosphatidylserine. The translocation of RACK1 from one subcellular location to another has been shown to mediate various cellular responses following a stimulus (Neasta *et al.*, 2012). However the mechanism of RACK1 localization to cellular membranes is not known. PIPs are clustered in distinct intracellular membranes and could each serve as a marker of different organelles. We propose therefore that one way by which RACK1 localize to different cellular membranes may be via its interaction with PIPs which in *D. discoideum* is

mediated by the polybasic stretch. Whether RACK1 proteins from other species are likewise able to bind to membrane lipids remains to be investigated.

G protein-linked signal transduction plays an essential role in the developmental program of *Dictyostelium* (Devreotes, 1994; Firtel, 1991; Wu *et al*., 1993). *D. discoideum* has twelve Gα subunits, one Gβ and one Gγ subunit. It is generally assumed that Gβ forms heterotrimers with the γ and all α subunits (Firtel, 1996). For RACK1 interactions with G protein heterotrimer and heterodimeric βγ subunits were reported (Dell *et al*., 2002, Chen *et al*., 2004; 2005). We describe here an interaction of DdRACK1 with Gα subunits 2, 4 and 8, as well as with the Gβ and Gγ subunits by yeast two-hybrid, co-immunoprecipitation and pull down experiments which in case of Gβ was further supported by confocal live cell imaging where RFP-RACK1 partially co-localized with GFP-Gβ. The mutant phenotypes that were observed after overexpression revealed roles in cell growth, chemotaxis and development which are processes in which Gα2, Gα4, Gα8 and Gβ also are implicated underlining a role for RACK1.

We have identified the novel RACK1 orthologue in *D. discoideum* (DdRACK1), which has significant sequence identity with other previously studied RACK1 species and similar biochemical features as bona fide RACK1 proteins. It contains an unusual polybasic region through which it can bind to cellular membranes uncovering a further mechanism how RACK1 can be targeted to membranes. At the biochemical level it interacts with several proteins among them ribosomal proteins, enzymes, cytoskeletal proteins and most notably heterotrimeric G proteins. Our genetic data provide evidence for a function as a scaffold protein. Upon overexpression we observe phenotypes that imply changes in signalling pathways regulated by the interacting G proteins. Taken all these results together, we propose that, through these interactions, RACK1 is involved in the regulation of several cellular processes (Figure 35).

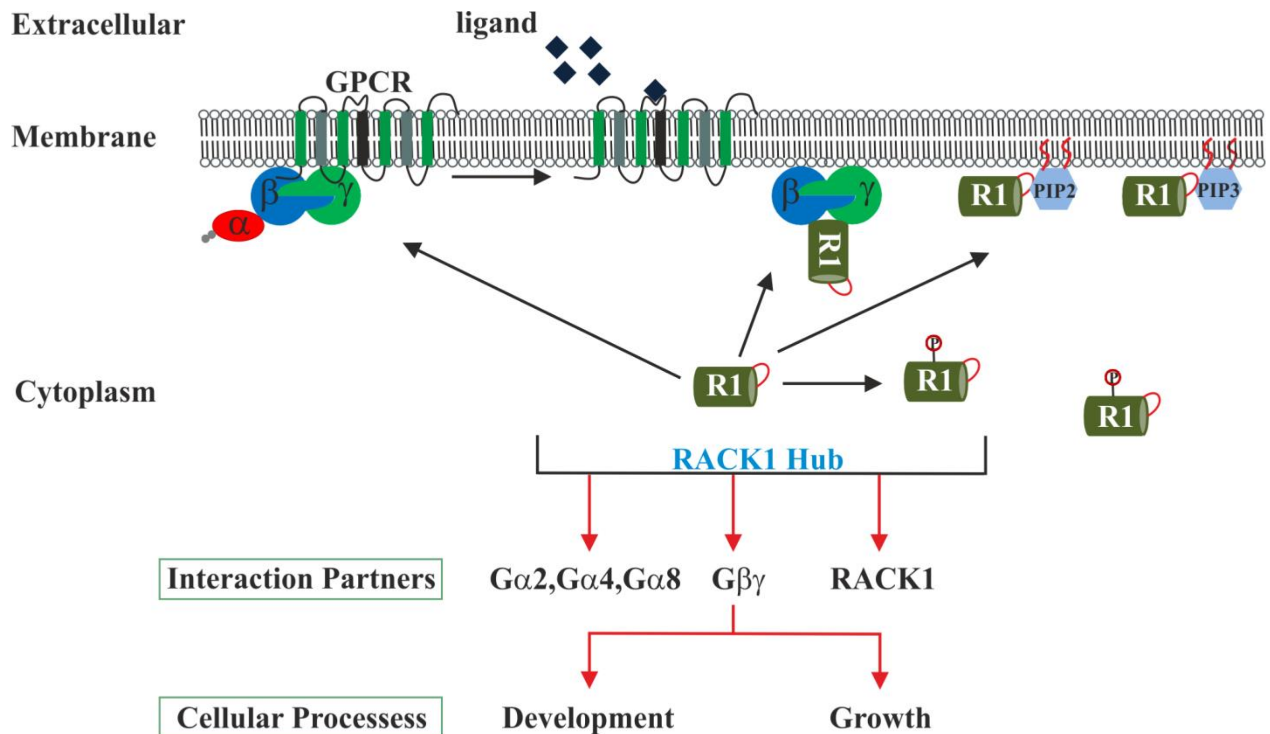


Figure 35 Model for DdRACK1 involvement in growth and development. The activation of Gβγ at the membrane initiates a series of events which modulates the production of PI(4,5)P_2 and PI(3,4,5)P_3 leading to the recruitment of DdRACK1 (RI). DdRACK1 binds to G proteins (which may be in their free or heterotrimeric state). This association could regulate G protein interaction with downstream effectors. Phosphorylation/dephosphorylation of DdRACK1 further could regulate its function. Interaction with the different partners confers DdRACK1 with roles in various cellular processes like growth, chemotaxis and development.

5 Summary

The receptor for activated C-kinase 1 (RACK1) is a conserved protein belonging to the WD40 repeat family of proteins. It folds into a beta propeller with seven blades which allow interactions with many proteins. Thus it can serve as a scaffolding protein and have roles in several cellular processes. We identified the product of the *Dictyostelium discoideum gpbB* gene as the *Dictyostelium* RACK1 homolog. The protein is mainly cytosolic but can also associate with cellular membranes. DdRACK1 binds to phosphoinositides (PIPs) in protein-lipid overlay and liposomes-binding assays. The basis of this activity resides in a basic region located in the extended loop between blades 6 and 7 as revealed by mutational analysis. Similar to RACK1 proteins from other organisms DdRACK1 interacts with G protein subunits alpha, beta and gamma as shown by yeast two-hybrid, pulldown, and immunoprecipitation assays. Unlike the *Saccharomyces cerevisiae* and *Cryptococcus neoformans* RACK1 proteins it does not appear to take over Gβ function in *D. discoideum* as developmental and other defects were not rescued in Gβ null mutants overexpressing GFP-DdRACK1. Overexpression of GFP-tagged DdRACK1 and a mutant version (DdRACK1mut) which carried a charge-reversal mutation in the basic region in wild type cells led to changes during growth and development. DdRACK1 interacts with heterotrimeric G proteins and can through these interactions impact on processes specifically regulated by these proteins.

Zusammenfassung

Der Rezeptor für aktivierte C-kinase 1 (RACK1) ist ein konserviertes Protein, das zur WD40 Repeat Familie gehört. Diese Wiederholungseinheiten sind siebenfach vorhanden und bilden einen siebenflügeligen β-Propeller, der die Interaktion mit vielen Proteinen ermöglicht. Rack1 kann als Gerüstprotein funktionieren und hat Rollen in verschiedenen zellulären Prozessen. Wir haben das Produkt des *Dictyosteliun discoideum gpbb* Gens als *Dictyostelium* RACK1-Homolog identifiziert. Das Protein ist im Wesentlichen cytosolisch, ist aber auch mit zellulären Membranen assoziiert. In Protein-Lipid Overlay und Liposomen-Binding Assays bindet DdRACK1 an Phosphoinositide (PIPs). Die Grundlage für diese Aktivität befindet sich in einer Lysin-reichen Aminosäuresequenz zwischen den Propellerblättern 6 und 7 wie eine Mutationsanalyse gezeigt hat. Ähnlich wie bei RACK1-Proteinen aus anderen Organismen, interagiert DdRACK1 mit den G-Protein-Untereinheiten Alpha, Beta und Gamma in Yeast Two-Hybrid, Pull down und Immunpraezipitations Analysen. Im Gegensatz zu den RACK1-Proteinen aus *Saccharomyces cerevisiae* und *Cryptococcus neoformans* scheint es nicht die Gβ Funktionen in *D. discoideum* zu übernehmen, da Entwicklungs- und andere Defekte in einer Gβ null Mutante nicht beseitigt wurden. Eine Überexpression des GFP- DdRACK1 Fusionsproteins und einer Mutantenversion (DdRACK1Mut), bei der die Lysine gegen Glutamatreste ausgetauscht waren, führte zu Änderungen bei Wachstum und Entwicklung. Wir schließen aus unseren Ergebnissen, dass DdRACK1 mit heterotrimeren G-Proteinen interagiert und durch diese Interaktion Einfluss auf Prozesse nehmen kann, die G-Proteine geregelt sind.

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Erklärung

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