Molecular aspects of GAPR-1 interactions with biological and model membranes

Moleculaire aspecten van GAPR-1 interacties met biologische membranen en modelmembranen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 20 mei 2008 des middags te 2.30 uur

door

Josse van Galen

geboren op 31 augustus 1979 te Utrecht

Promotor:	Prof.dr. J.B. Helms
Co-promotoren:	Dr. D.V. Kaloyanova Dr. J.J. Batenburg

Contents

Layout by H. Otter
Cover design by M. Brouwer
Printed by Ridderprint, Ridderkerk, The Netherlands

ISBN: 978-90-393-4813-0

Chapter 1

General Introduction

Based on "Pathogens Exploit the Complexity of the Lipidome"
Invited Review in preparation

1 Lipids and biological membranes

One of the prerequisites of life is the ability to compartmentalize biological processes. Hereby, enzymes and their substrates are concentrated and their reaction products are contained. In addition, for prevention of futile cycles in e.g. metabolic pathways, higher organisms can segregate components in separate compartments. Biological compartments are required to have dynamic properties, as waste products need to be disposed of and new metabolites need to be attracted and contained. Biological membranes fulfill these requirements as they are semi permeable: ion fluxes and passage of molecules are restricted and tightly regulated. The basic structure of biological membranes that enables the aforementioned tasks is that of a bilayer of lipids (Fig. 1A). Lipids are loosely defined as biological molecules with hydrophobic or amphipathic properties that render them soluble in organic solvents (Fahy et al., 2005). Despite the fact that this definition includes thousands of different chemical structures, a more defined classification seemed unnecessary for a long period of time, as lipids were believed to have two general functions: a structural role in biomembranes and an energy-storage role in cells (lipid droplets) and organisms (lipoproteins). Animal membranes are generally comprised of polar lipids, cholesterol and proteins. Polar lipids, mainly glycerophospholipids and sphingolipids, form a bilayer and are the main building blocks of a membrane (Fig. 1B). Glycerophospholipids consist of a glycerol backbone to which two fatty acids are bound via an ester bond at the *sn-1* and *sn-2* position and a phosphate-containing headgroup at the *sn-3* position. Sphingolipids consist of a sphingosine backbone to which an acyl chain is linked via an amide bond and a headgroup is bound via an O-linkage. Neutral lipids form a third major group, which contains cholesterol, diacylglycerol (DAG) and triacylglycerol (TAG) (Fig. 1C). Cholesterol is a sterol lipid that is not capable of forming lipid bilayers, but it regulates membrane fluidity when incorporated into the lipid bilayer. DAG and TAG consist of a glycerol backbone to which two or three acyl chains are coupled, respectively. Whereas DAG can be part of the lipid bilayer, TAG is too hydrophobic for water exposure and therefore it does not form bilayers, but localizes between lipid bilayers or localizes to lipid droplets instead.

1.1 Functions of lipids

During the last two decades we have come to realize that, in addition to being building blocks of membranes and being energy storage molecules, lipids have a multitude of different and essential functions in the cell.

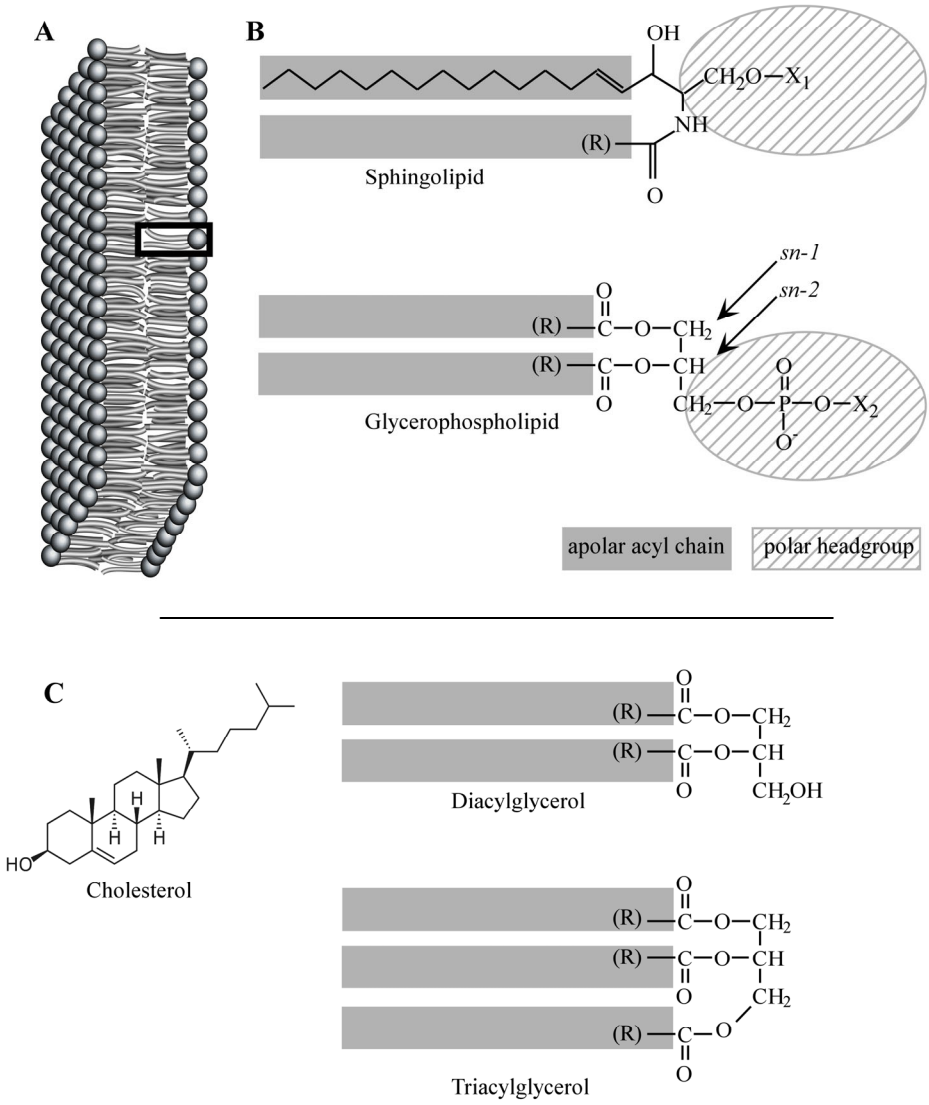


Figure 1
(A) Schematic drawing of a membrane consisting of a lipid bilayer. A single polar lipid is highlighted in the box, and which is shown in more detail in Fig. 1B. (B) Molecular structure of a sphingolipid and a glycerophospholipid. R represents an acyl chain, that can vary in length and degree of saturation. X_1 and X_2 represent the headgroup of a sphingolipid or glycerophospholipid respectively. The apolar acyl chains and polar headgroups are depicted in solid or striped gray, respectively. (C) Molecular structure of cholesterol, diacylglycerol and triacylglycerol

First indications for the involvement of lipids in cellular signaling comes from the groundbreaking work of Irvine and Berridge in the 1980s showing that hydrolysis of phosphoinositides generates second messengers (Berridge and Irvine, 1989). Since the identification of the phosphatidylinositol transfer protein as an essential factor for protein trafficking from the trans-Golgi network (TGN) in yeast in the early 1990s (Bankaitis et al., 1990), abundant evidence implicates the involvement of lipids in the regulation of membrane traffic (Haucke and Di Paolo, 2007). Lipids have also been implicated in the generation of lateral heterogeneity in biological membranes, creating membrane domains that are often referred to as lipid rafts (Simons and Vaz, 2004)

By specific recruitment of proteins and lipids to these domains while excluding others, many biological processes such as cell migration, the immune response, and the cell cycle are affected or regulated.

Thus, lipids have a multitude of functions in many biological processes and we start to understand the long mysterious reason why nature synthesizes thousands of different lipids. The involvement in a variety of host cell functions makes lipids an attractive target for pathogens. Intracellular pathogens typically bind to the host cell membrane, are internalized by a phagocytic(-like) process and are degraded by the hostile environment in subsequent endocytic compartments. Some pathogens, however, escape degradation by interacting with the cellular machinery at any one of these steps, allowing their survival and multiplication. Today, there are many indications for an important role of lipids in various stages of host-pathogen interactions. One of the first examples for the involvement of lipids in host-pathogen interactions comes from the bacterium *Vibrio cholerae* that secretes the enterotoxin cholera toxin. The receptor of the toxin is a lipid termed GM1 (King and Van Heyningen, 1973). This protein-lipid interaction results in the formation of a membrane pore, ultimately resulting in severe diarrhea. Below we will review a few examples of pathogens that take advantage of the complexity of the lipidome. As will be described, pathogens have devised sometimes ingenious strategies to modify the cellular lipid homeostasis, allowing them to divert cellular processes.

1.2 Pathogens use host lipids for biogenesis

Pathogens often capture lipids from their host cells and use them to their own benefit. One of the most basic interests of pathogens in these lipids is their use in the biogenesis of the pathogen. Enveloped viruses, such as influenza A virus and HIV, use host lipids to form their envelope. Lipids are among the few components in

viruses that are generally not synthesized by enzymes encoded by the viral genome. During biosynthesis, viruses select lipids from the host cell, resulting in enrichment of certain lipid classes on viruses (Brugger et al., 2006; Cluett and Machamer, 1996). The intracellular parasite Toxoplasma Gondii harvests cholesterol and other host lipids and uses them as precursors for biosynthesis of parasite membranes (Charron and Sibley, 2002). Usage of host lipids may be a common phenomenon for apicomplexan parasites as incorporation of fatty acids is observed in T. Gondii and Plasmodium Falciparum (Mazumdar and Striepen, 2007). Another interesting use of lipids by T. Gondii is the gathering of host cholesterol in the rhoptry, a vacuolar organelle of these parasites. T. Gondii discharges the cholesterol from the rhoptry after entry into cells via caveolae. Cholesterol is also required for parasite entry (Coppens and Joiner, 2003), and it appeared that cholesterol from the host cell is important for entry but not the cholesterol derived from the rhoptry. Why T. Gondii accumulates host cholesterol remains enigmatic.

Hepatitis C virus (HCV) is able to manipulate lipid biosynthesis by regulation of lipid enzyme expression in the host (Su et al., 2002). HCV replication is dependent on the fatty acid composition of the host. Saturated acyl chains enhance both transcription of HCV RNA and replication. In contrast, polyunsaturated acyl chains, or inhibition of fatty acid biosynthesis leads to inhibition of HCV replication (Kapadia and Chisari, 2005).

Pathogens do not only use lipids for biogenesis, but also for the generation of energy. An example of this is the consumption of lipid acyl chains by Mycobacteria using isoforms of the glyoxylate cycle enzyme isocitrate lyase for fatty acid catabolism (Munoz-Elias and McKinney, 2005).

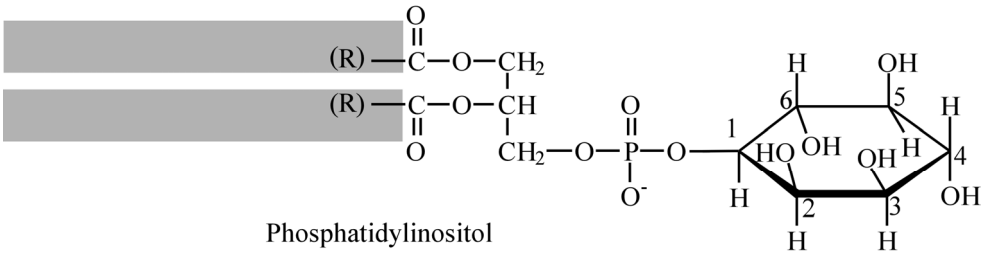
1.3 Phosphoinositide signaling

Lipids play a major role in cellular signaling (De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006; Hannun and Obeid, 2008). In signaling cascades, lipids can be synthesized *de novo* or enzymes can transform bulk lipids, which play a structural role in membranes, into signaling lipids as second messengers. In general, signaling lipids have a high turnover and are present in minute amounts. This does not exclude the possibility that these signaling lipids (transiently) appear at high concentrations in subdomains of the membrane. For instance, sphingomyelin (SPH), a structural bulk lipid, can be transformed in sphingosine-1-phosphate (S1P) (Hannun and Obeid, 2008). Intermediates in the transformation from SPH to S1P and S1P itself play a signaling role in various cellular processes [reviewed in (Hannun and Obeid, 2008)].

Phosphoinositides are another important class of signaling lipids, as they are involved in numerous cellular signaling cascades. Phosphatidylinositol (Fig. 2A) can be phosphorylated at its 3, 4 and 5 position in all possible combinations, leading to 7 different phosphoinositide species. Phosphorylation at other positions is also possible but less common. Phosphoinositides are involved in cellular signaling via two main mechanisms: 1) hydrolysis of phosphoinositides yields second messengers, enabling them to transmit signals; and 2), Phosphoinositides serve as a docking site for proteins with domains that recognize specific phosphoinositides (Lemmon, 2008). As the generation or localization of phosphoinositides is organelle-specific (Fig. 2B), phosphoinositides help define the identity of an organelle by recruitment of specific proteins. By binding to effectors, phosphoinositides are also able to regulate cytoskeletal rearrangement and membrane remodeling. Therefore, phosphoinositide metabolism plays a central role in receptor-mediated endocytosis and phagocytosis. In professional phagocytes, PIP5KinaseI is activated upon host-pathogen interaction resulting in the phosphorylation of PI(4)P and yielding $PI(4,5)P_2$ (Coppolino et al., 2002; Yeung et al., 2006). $PI(4,5)P_2$ is subsequently modified by either dephosphorylation of the 4 or 5 phosphate, by phosphorylation at the 3 position creating $PI(3,4,5)P_3$, or by removal of the headgroup by PLCγ.

Formation of PI(3)P is also essential, as inhibition of PI3 kinases inhibits phagosomal maturation. This is a very dynamic process, as multiple waves of PI(3)P attract effector proteins such as EEA1 and Hrs to the phagosome, which is important for subsequent signaling processes (Chua and Deretic, 2004). In later stages of phagosome maturation, $PI(3,5)P_2$ is generated through phosphorylation of PI(3)P by PIKfyve, an enzyme that is also involved in formation of the late endosome or multivesicular body (Ikonomov et al., 2003). As a result, PI(3)P effector proteins are released from the membrane and $PI(3,5)P_2$ effector proteins will be recruited to the phagosomes. Thus, phosphoinositides contribute to the dynamic process of the entire maturation process, which is characterized by multiple cycles of membrane binding and release of proteins (Rogers and Foster, 2007).

A



B

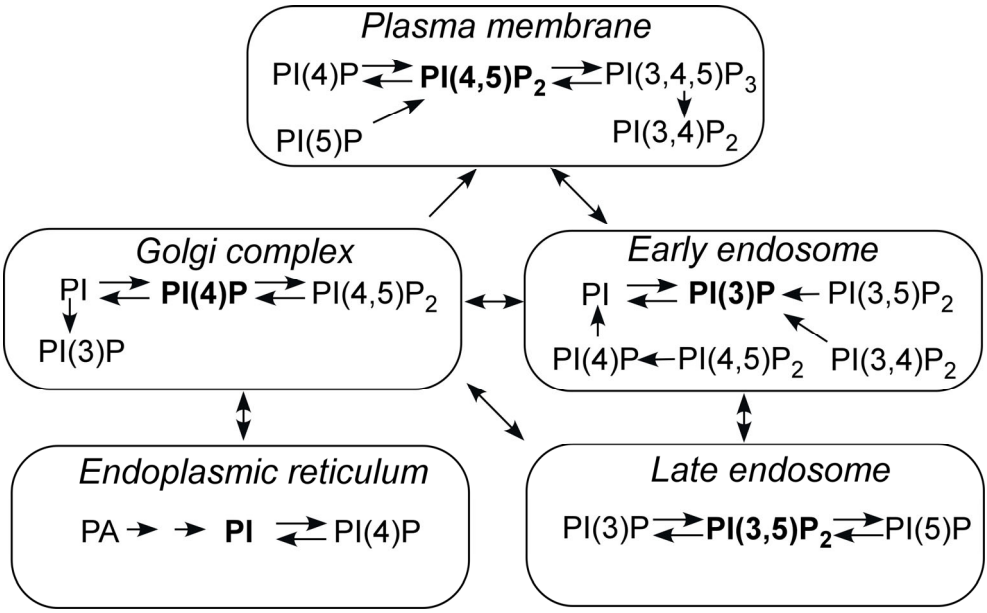


Figure 2
(A) Molecular structure of phosphatidylinositol. Positions in the inositol ring are numbered 1-6. R represents an acyl chain with varying length and degree of saturation. (B) Predominant subcellular localization of phosphoinositide species. Phosphorylation/dephosphorylation events occurring at each subcellular compartment are illustrated. In the ER, the conversion of phosphatidic acid (PA) to phosphatidylinositol (PI) involves more than one reaction. Adapted from Di Paolo and De Camilli (Nature, 2006).

1.4 Manipulation of phosphoinositide signaling by pathogens

Phosphoinositides are important regulators of phagocytosis and hence, interesting targets for pathogens to manipulate this cellular process. After uptake by

phagocytosis, pathogens avoid degradation by interfering with the maturation or escape from the phagosome. There is an increasing number of pathogens known to take over phosphoinositide signaling in the host cell enabling them to replicate in an optimal environment. By interference with phosphoinositide metabolism, pathogens can affect either the initial uptake process (A) or the phagosomal maturation process (B). Pathogens are able to secrete phosphoinositide effectors via their type III or IV secretion systems, or manipulate phosphoinositide signaling otherwise (C).

(A) *L. monocytogenes* secretes InlB via its type III secretion system, which stimulates PI(3) kinase. By generation of PI(3)P and its derivatives the uptake of *L. monocytogenes* increases. *Pseudomonas aeruginosa* can only enter cells via basolateral membranes. To enable its uptake at the apical membrane, *P. aeruginosa* stimulates a PIP3 kinase at the apical membrane to increase local phosphoinositide levels that are phosphorylated at the 3 position (Kierbel et al., 2005). Formation of $PI(3,4,5)P_3$ leads to transformation of apical membrane into basolateral membrane in polarized MDCK cells (Gassama-Diagne et al., 2006). This transformation makes the apical membrane accessible for *P. aerigunosa* entry (Kierbel et al., 2007).

(B) After uptake, pathogens may disrupt phosphoinositide signaling by secretion of lipid modification enzymes, allowing them to interfere with the maturation process. As these enzymes lead to propagation of the infection, they are often regarded as virulence factors (Hilbi, 2006). To disrupt the host response, *Salmonella typhimurium* secretes SigD (also known as SopB) via a type III secretion system. SigD is a phosphatase that dephosphorylates inositol phosphates and phosphoinositides at various positions (Zhou et al., 2001). In cells, SigD preferably removes phosphates from 4 and 5 positions of phosphoinositides. During phagocytosis, SigD dephosphorylates $PI(4,5)P_2$, (Terebiznik et al., 2002), $PI(3,4,5)P_3$ and $PI(3,5)P_2$ (Gruenberg and van der Goot, 2006; Hernandez et al., 2004). As a result, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ effectors are not recruited to phagosomes, and therefore phagosomes are diverted from the endocytic pathway. This results in a non-acidic phagosome intermediate that is optimal for replication of the *Salmonella*. *Shigella flexneri* secretes IpgD, which is a phosphatase that dephosphorylates $PI(4,5)P_2$ to PI(5)P. In this way the actin cytoskeleton, in which $PI(4,5)P_2$ is a key regulator (Qualmann and Kessels, 2002), is affected (Niebuhr et al., 2002), thereby disrupting phagosome transport and maturation.

Pathogens can also manipulate the phosphorylation at the 3 position of phosphoinositides to disrupt phagosome maturation. Enteropathogenic *E.coli* secretes, via its type III secretion system, EspF to block host PI(3) kinase activity

(Celli et al., 2001; Quitard et al., 2006). In contrast, ulcerogenic *Helicobacter pylori* strongly activate PI(3) kinases to disrupt actin cytoskeleton regulation, which causes a delayed phagocytosis (Allen et al., 2005). *M. tuberculosis* secretes via an unknown mechanism SapM, a lipid phosphatase that hydrolyzes PI(3)P, which inhibits phagosome-late endosome fusion *in vitro*, thereby arresting phagosomal maturation (Vergne et al., 2005).

(C) In addition to modification of host cell phosphoinositides, *M. tuberculosis* can disrupt phosphoinositide signaling by molecular mimicry of phosphoinositides. It carries mannosylated forms of phosphatidylinositol at its surface that resemble phosphoinositides and which can be inserted into the phagosomal membrane. *M. tuberculosis* uses mannose-lipoarabinomannan (ManLAM) to disrupt EEA1 and Vps34 (PI3K) activities, which regulate endosomal trafficking events downstream of the small GTPase Rab5 (Fratti et al., 2003; Rosenberger and Finlay, 2003). In addition, phosphatidylinositol mannoside (PIM), a precursor of LAM, stimulates EEA1 to enhance fusion of phagosomes with early endosomes instead of fusion with lytic organelles (Vergne et al., 2004). In summary, *M. tuberculosis* uses various strategies to evade an adequate immune response via lipid manipulation. The multitude of ways in which pathogens in general interfere with phosphoinositide signaling imply that this signaling pathway is a common target for pathogens to escape from degradation in the lysosome.

1.5 Lipid rafts

The fluid Mosaic model (Singer and Nicolson, 1972) describes membranes as lipid bilayers with free lateral diffusion of lipids and proteins, often envisioned as a 'sea' of randomly distributed lipids and proteins. More recent and refined models propose that lipids are not distributed randomly in membranes (Lisanti and Rodriguez-Boulan, 1990; van Meer and Simons, 1982) but that membranes are compartmentalized as well. Possibly, small domains termed lipid rafts are formed that contain densely ordered lipids. This gives rise to locally altered membrane properties that may exclude or attract proteins. Transmembrane and prenylated proteins are described to be excluded from rafts. In contrast, proteins that are dually acylated and GPI-anchored proteins are concentrated in rafts at the cytosolic leaflet and the exoplasmic/luminal leaflet of the membrane, respectively (Simons and Ikonen, 1997). By selective inclusion and exclusion of proteins, cellular processes are concentrated in rafts. Rafts are implicated in many cellular processes such as signaling, trafficking of vesicles to and from the plasma membrane (Ikonen, 2001;

Mukherjee and Maxfield, 2000), cell adhesion (Krauss and Altevogt, 1999; Lacalle et al., 2002), directed cell movement (Pierini and Maxfield, 2001), protein sorting (Manes et al., 1999), generation of cell polarity (Manes et al., 1999), and pathogen entry and exit [section 1.6 of this chapter and (Simons and Ikonen, 1997; Yoshizaki et al., 2007)].

Domain formation *in vitro* is driven by differences in melting temperature of lipids and weak Van der Waals interactions between lipids. Lipids can be in a solid phase (S_o) in which acyl chains are stretched and closely packed, thereby restricting movement by diffusion, or in a liquid disordered phase (l_d) in which acyl chains can bend and lipids can diffuse freely. Lipids go through the phase transition from the S_o phase to the L_d phase at their melting temperature (T_m). However, when lipid mixtures contain cholesterol, this may intercalate between the acyl chains, thereby affecting the phase of the lipid: below T_m cholesterol fluidizes lipids in the S_o phase and above T_m it rigidifies lipids in the l_d phase. In this way a transition phase, the liquid ordered phase (l_o), exists, which has mixed properties of the S_o and the l_d phase (Ipsen et al., 1987). In the l_o phase, the lateral diffusion coefficient is reduced as compared to the l_d phase (Almeida et al., 1993) and the conformational order of the lipid acyl chains is similar to that of the S_o phase (Gally et al., 1976; Simons and Vaz, 2004). In model membranes containing cholesterol, the l_o and l_d may coexist at 37 °C, but form different domains. Lipids that interact with cholesterol and possess a relatively high lateral cohesion can enter the l_o phase. Cholesterol interacts efficiently with sphingolipids (Sankaram and Thompson, 1990) but also with glycerolipids with a saturated *sn-1* acyl chain (Brown and London, 1998; Rog and Pasenkiewicz-Gierula, 2001), thus l_o domain formation is possible for both lipid classes. The affinity of lipids to form an l_o phase is determined by the lipid backbone, the acyl chain length, and degree of saturation. Lipids with a sphingosine backbone can interact with each other via hydrogen bonds. For example sphingomyelin, a sphingolipid with a phosphocholine headgroup, contains both hydrogen donors (-NH and –OH, Fig. 1A, sphingolipid) and acceptor (esteric O, Fig. 1A, sphingolipid). Phosphatidylcholine, a glycerophospholipid with a choline headgroup, only has hydrogen acceptors (esteric O's, Fig. 1A, glycerophospholipid) (Boggs, 1987). Acyl chain length affects l_o phase partitioning, as addition of 2 CH_2 units results in doubling or tripling of l_o phase partitioning (Palestini et al., 1995). For l_o phase participation, saturated acyl chains are preferred as their flexibility facilitates ordering, whereas a rigid kink in unsaturated chains frustrates ordering. Glycosphingolipids *in vivo* contain generally longer and more saturated acyl chains than glycerolipids (Dickson, 1998; Eisenkolb

et al., 2002). Therefore, membranes with a high glycosphingolipid and cholesterol content are predicted to contain a relatively high amount of domains in the l_o phase.

Membrane domains form spontaneously in model membranes. However, cellular membranes are much more complex than model membranes. Many different lipids are present with different lipid backbones (glycerol or sphingosine), a large number of head groups, which can then be differentially glycosylated or phosphorylated, and hydrophobic tails that can vary in length and degree of saturation. In cellular membranes, lipids are therefore found in more than 1000 different combinations (Sud et al., 2007; van Meer, 2005). As a result, domains that are formed are very heterogeneous in size (10-200 nm), with highly dynamic properties in terms of size and composition, and domains can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Jacobson et al., 2007). A probe that detects GM1, a glycosphingolipid, labels other domains in cells than lysenin, which detects sphingomyelin (Hullin-Matsuda and Kobayashi, 2007; Kiyokawa et al., 2005). Lipid rafts may not only attract specific proteins, but proteins may also attract lipids, thereby forming a very small lipid raft or "lipid shell" (Helms and Zurzolo, 2004). For instance, influenza hemagglutinin (HA) was proposed initially to be recruited to lipid rafts (Takeda et al., 2003). However, more recent data suggest that HA itself may form rafts (Hess et al., 2005).

When raft domains are crosslinked with each other, raft proteins are separated from the non-raft proteins (Harder et al., 1998). This mechanism may be used in signaling. Lyn, a raft-resident signaling protein, can activate itself by phosphorylation and is localized on small rafts. When the IgE receptor (FcεRI) is activated, it enters rafts and crosslinks them into larger rafts, thereby sequestering Lyn from inactivating non-raft phosphatases (Brown, 2006). This mechanism may prolong Lyn activity and enhance cellular signaling.

Caveolae are a subset of lipid rafts at the plasma membrane and are characterized by their flask-like morphology. Caveolae are defined by the presence of the cholesterol-binding protein caveolin, which forms large oligomer complexes in rafts. Caveolin is required for formation of caveolae as caveolin-null cells are devoid of caveolae (Drab et al., 2001). Caveolae are enriched in signaling molecules such as tyrosine kinases, MAP kinases, G-coupled receptors, adenylyl cyclase and lipid signaling intermediates (Anderson, 1998; Lisanti et al., 1995), strongly implicating caveolae in signaling.

Non-caveolar rafts do not have a defined morphology, and their dynamic properties, such as their transient interactions with proteins, make it difficult to

investigate lipid rafts. Only few techniques are available to harvest membrane microdomains. The most frequently used method is solubilization of membranes by nonionic detergents. This method exploits the property of ordered membranes to exclude detergent, whereas non-raft domains are solubilized upon detergent addition. As the membrane domains are enriched in lipids, they can be isolated by flotation after density gradient ultracentrifugation. However, many pitfalls are known, such as aggregation of membranes by detergents (Madore et al., 1999), which lead to isolation of rafts that are artificially large (Glebov and Nichols, 2004). Furthermore, detergent-insoluble domains are formed in model membranes by the detergent itself (Heerklotz, 2002) and phosphoinositides could be relocated in insoluble membranes by detergents (Munro, 2003; van Rheenen et al., 2005). Solubilization by some detergents, such as Triton X-100, requires chilling, which may expand the l_o domain size. Moreover, the isolated proteins that are identified in the isolated detergent-insoluble fraction are dependent on the choice of detergent (Lichtenberg et al., 2005; Schuck et al., 2003). Therefore, raft localization of proteins that are isolated by detergent extraction need to be confirmed by additional methods, some of which are reviewed elsewhere (Jacobson et al., 2007).

1.6 Pathogens exploit lipid rafts for entry and immunomodulation

Concentration of receptors or oligomerization of proteins in lipid rafts may increase the binding affinity of the pathogens to host cells (van der Goot and Harder, 2001) or may allow pathogens to disrupt signaling processes in rafts, leading to enhanced uptake or prolonged intracellular survival. Uptake of SV40, a non-enveloped virus, is raft dependent and leads to the recruitment of caveolin (Pelkmans et al., 2001). A requirement for uptake of SV40 is binding to MHC class I, which is enriched in caveolae. Interestingly, SV40 can also enter cells in the absence of caveolae and localize to caveosome-like organelles. However, also in that case the uptake of SV40 is still raft dependent (Damm et al., 2005). It appears that enveloped viruses also select raft-like domains to exit from cells (Nguyen and Hildreth, 2000), as viral membranes are Triton X-100 resistant (Brugger et al., 2006) and contain raft markers (GM1, Thy-1 and CD59).

Also the action of many bacterial toxins is dependent on lipid rafts. Cholesterol is the receptor for streptolysin and sphingomyelin for lysenin. *Vibrio cholerae* cytolysin requires cholesterol and sphingomyelin for efficient oligomerization and channel formation (Fivaz et al., 1999). Cholera toxin clusters into pentameric structures upon binding to the lipid raft marker GM1 (Eidels et al., 1983; Merritt et al., 1994). It

binds to detergent-resistant membranes (Wolf et al., 1998) and intoxification could be achieved by interference with raft integrity (Orlandi and Fishman, 1998). Thus, membrane microdomains create a good environment to cluster/oligomerize pore-forming proteins. Evidence for such a role of rafts in the oligomerization process has been described for the toxin aerolysin. The receptor-bound toxin distributes in punctate structures on the plasma membrane of living cells and is enriched in detergent-insoluble microdomains (Abrami et al., 1998).

L. monocytogenes makes pores with a cytolysin (listeriolysin O) (Tweten, 2005). The cholesterol-dependent listeriolysin O aggregates rafts via oligomerization (Gekara et al., 2005). The pore formation disrupts phagosome maturation and signals for prolonged survival of the host cell, which provides *L. monocytogenes* more time to divide (Gruenberg and van der Goot, 2006).

Pathogens also exploit the signaling capacity of rafts to signal for organization of pathogen uptake. For example, pathogenic *Brucella* bacteria secrete cyclic β-1,2-glucan that weakly extracts cholesterol from membranes, thereby disrupting rafts. As a consequence, cyclic β-1-2-glucan inhibits phagosome-lysosome fusion (Arellano-Reynoso et al., 2005). The parasite *Leishmania donovani* may affect raft integrity at phagosomes after uptake into macrophages by use of the GPI-anchored lipophosphoglycan on its surface (Dermine et al., 2005). Interestingly, lipophosphoglycan changes the raft distribution over phagosomal membranes rather than changing the raft protein content of phagosomes. Therefore, despite the presence of signaling proteins on phagosomes, signal transduction for phagolysosomal maturation is inhibited.

1.7 Lipid rafts in the secretory pathway

Lipid rafts were initially identified at the plasma membrane. The high concentration of sphingolipids and cholesterol at the plasma membrane makes it ideally suited for raft formation (Ahmed et al., 1997). Estimations of relative raft amounts on the plasma membrane vary from approximately 13 % (Schutz et al., 2000) to 35% (Prior et al., 2003). On endothelial cells, caveolae account for ~30% of the cell surface (Goligorsky et al., 2002).

Rafts also occur on intracellular membranes, either by (i) internalization of rafts from the plasma membrane or by (ii) *de novo* generation of rafts along the secretory pathway. (i) Cells can internalize caveolae or non-caveolar lipid rafts and sort them to caveosomes or endosomes, respectively. In recycling endosomes lipid rafts may be involved in protein sorting (Mukherjee and Maxfield, 2000). (ii) *De novo*

generation of lipid rafts likely depends on a gradient of cholesterol and sphingomyelin, of which the concentrations increase from the endoplasmic reticulum to the plasma membrane. Cholesterol is synthesized at the endoplasmic reticulum (ER) (Reinhart et al., 1987). The Golgi apparatus is the major site of sphingolipid biosynthesis within the cell (Futerman et al., 1990; Jeckel et al., 1992). In the Golgi a gradient of cholesterol exists across the cisternae, with higher levels at the *trans* side (Pagano et al., 2000). To explain this gradient, it was proposed that cholesterol-rich membrane domains are selectively transported forward through the Golgi toward the plasma membrane (Bretscher and Munro, 1993). Lipid rafts could also be involved in maintenance of the distinct lipid compositions of the plasma membrane and organelles of the secretory pathway that are maintained in the face of membrane traffic in both directions (Mukherjee and Maxfield, 2000). Although in the ER no sphingolipids are present, rafts have been detected (Bagnat et al., 2000).

1.8 Golgi-derived detergent-insoluble complexes

Lipid rafts have also been isolated as detergent-insoluble complexes from the Golgi complex and characterized in terms of protein and lipid composition (Gkantiragas et al., 2001). As these Golgi lipid rafts are insoluble in Triton X-100, they were named Golgi-derived Insoluble Complexes (GICs). Analysis of the protein composition of GICs revealed that GICs contain a relatively simple set of ten major proteins (Fig. 3) (Gkantiragas et al., 2001). All proteins have a cytosolic orientation except for caveolin-1 and GREG (Fig. 3). Caveolins form a hairpin loop in the membrane and GREG has recently been shown to have a Golgi luminal orientation (Li et al., 2007). The topological orientation of the GIC proteins at Golgi membranes is schematically shown in Fig.3. When cells were treated with Brefeldin A, the GIC resident proteins GREG, flotillin-1 and GAPR-1 were relocated, which suggests that GICs are localized to the early Golgi complex (Eberle et al., 2002; Gkantiragas et al., 2001). Golgi localization of the rafts may imply that they are precursors for plasma membrane rafts that are transported via the secretory pathway. Alternative possibilities include a role in maintenance of the Golgi structure and function, as changing the Golgi cholesterol levels affects Golgi transport (Stueven et al., 2003) and results in Golgi vesiculation (Grimmer et al., 2000; Hansen et al., 2000).

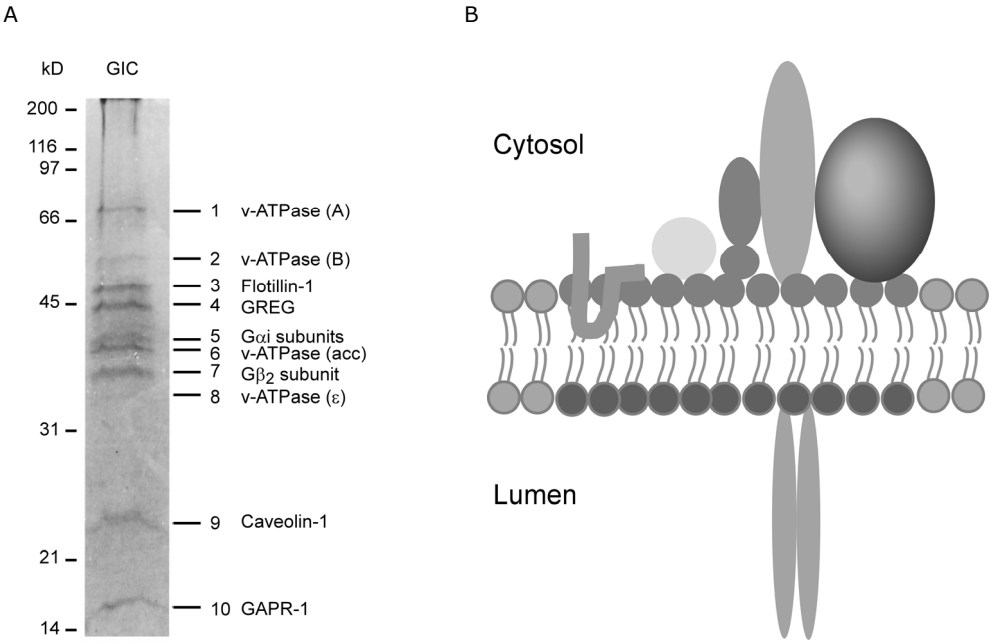


Figure 3
(A) Protein content of GICs, analyzed by SDS-PAGE and subsequent coomassie blue staining. Adapted from Gkantiragas *et al.* (Mol Biol Cell, 2001). (B) Putative topology of GIC proteins at the Golgi membrane. Caveolin-1 forms a hairpin loop in the membrane (Monier et al., 1995). GREG is a GPI-anchored protein residing in the Golgi lumen (Li et al., 2007). All other proteins (schematically depicted in different shades) have a cytosolic orientation. The topology of flotillin-1 is not clear and this putative transmembrane protein has been omitted from the cartoon.

The GIC protein GREG has been shown to be essential for Golgi maintenance as well, as protein secretion and Golgi morphology were affected upon knockdown of this protein (Li et al., 2007). Various GIC proteins suggest a role of GICs in signaling. Subunits of heterotrimeric G proteins, especially the αi subclass of G proteins and β subunits, are highly enriched in GICs (50-100 fold) as compared to their donor Golgi membranes (Gkantiragas et al., 2001). This suggests that GICs form signaling platforms, possibly involved in maintenance of Golgi structure and function (Helms et al., 1998). Also the other proteins that are located in GICs imply a signaling function of Golgi rafts. Flotillin-1 is a raft marker (Bickel et al., 1997; Volonte et al., 1999) that might be important for transmembrane signaling in rafts (Li et al., 2007).

The smallest GIC protein (17 kDa) was classified as a mammalian homologue of group 1 of plant pathogenesis-related proteins (PR proteins) and was named Golgi-

Associated Plant Pathogenesis-Related protein 1 (GAPR-1) (Eberle et al., 2002). GAPR-1 will be further discussed below (section 2.1).

The Golgi apparatus exchanges membranes with several other subcellular organelles, including endosomes, caveosomes, autophagosomes, and lipid droplets (Legesse-Miller et al., 2000; Litvak et al., 2002; Mallard et al., 1998; Nichols et al., 2001). It is not known whether GICs can also relocate to these membranes, but some Golgi raft proteins have been found to relocate to other organelles. For example, GAPR-1 localizes to exosomes (Adachi et al., 2006; Pisitkun et al., 2004). Prior to entering exosomes, GAPR-1 must be transported from the Golgi to the limiting membrane of a late endosome (Stoorvogel et al., 2002). It is not known whether relocation of GAPR-1 to endosomes occurs via the cytosol or via membrane intermediates. Several GIC components, including flotillin-1 (Garin et al., 2001) and GAPR-1, are found on phagosomes (Burlak et al., 2006; Dermine et al., 2001). Flotillin-1 in phagosomes is not derived from the plasma membrane, implying a relocation of flotillin-1 and possibly other GIC proteins from the Golgi complex.

2. Plant Pathogenesis related proteins

GAPR-1 is highly homologous to PR-1 proteins in plants. The function of PR-1 proteins in plants may indicate a function for GAPR-1 in mammals. Like animals, plants have a defense system against pathogens such as oomycetes, fungi, bacteria and viruses. Plants constitutively secrete antimicrobial proteins and compounds such as phytoanticipins and have, unlike animal cells, a cell wall to keep potential invaders outside. When pathogens succeed in invading, plants can induce pathogen resistance. Upon infection, plants start to secrete phytoalexins, which are plant antibiotics. Locally, plant cells can respond by deposition of callose, lignin and suberin, which reinforces cell walls (Hammond-Kosack and Jones, 1996). In addition, they may respond with an oxidative burst. This leads to direct killing of the pathogen, further reinforcement of the cell wall by protein crosslinking, or to death of the plant cell, which limits further spread of the pathogen. In the case that plant cells survive the oxidative burst, the reactive oxygen species enhance the transcription of resistance effector proteins and compounds (Lamb and Dixon, 1997).

Plants secrete hormones for regulation of systemic resistance. In case of invasion by non-pathogenic organisms, plants secrete jasmonic acid (JA) and ethylene (ET), which leads to Induced Systemic Resistance (IST). This is a mild form of resistance

that is not harmful to the plant itself (van Loon and van Strien, 1999). When plants are invaded by pathogens, they regulate their immune response by salicylic acid (SA) and this induces Systemic Acquired Resistance (SAR). SAR is potentially harmful for the plant, as this may lead to oxidative bursts and thus cell damage. Regulation of the SA response is not clear, but activated oxygen species may play a role in this process.

By regulation of plant hormones or reactive oxygen species, plants induce expression of pathogen related proteins (PR). They have originally been defined as being absent in uninfected plants but accumulating in large amounts after infection (van Loon and van Kammen, 1970). PR proteins have been grouped in 17 families (van Loon et al. 1994), and recently an 18^{th} family was suggested (van Loon et al., 2006). There are subdivisions within the described classes between acidic and basic PR proteins. The acidic proteins are usually secreted out of plants, whereas the basic proteins are secreted into the plant vacuole (Heil and Bostock, 2002) and are often induced by JA or SA. Several PR proteins are enzymes that directly attack the invader by hydrolysis of structural components. However, of some protein families the function is unknown and not all families are represented in all plant species. Unlike vertebrates, plants cannot build up an adaptive immune response or acquire an immunologic memory. Nevertheless, they can build up SAR by previous small infections. Plant immunity appear reminiscent of the innate immune response in vertebrates in several aspects (Dangl and Jones, 2001; Nurnberger et al., 2004)

2.1 GAPR-1

GAPR-1 shows homology to proteins from the PR-1 family. This family consists of small proteins that resist denaturation by extreme pH values and that are relatively resistant to proteolysis. PR-1 proteins are generally used as marker proteins for SAR and are upregulated by SA. Their upregulation is however not limited by pathogen infection, as they are upregulated during stress responses such as high UV exposure or oxidative stress. All PR-1 proteins are in the range of 14-17 kDa and contain 4 α-helices and 4 β-strands and six conserved cysteines and often have very basic or acidic pI values. For PR-1 proteins, anti-fungal activity (Niderman et al., 1995) and anti-oomycete (Alexander et al., 1993) has been described. Other publications concerning animal PR-1 homologues imply a serine protease activity (Milne et al., 2003). The highly conserved histidine and glutamate pairs of the PR-1 family have also been proposed to represent a catalytic tetrad, although the arrangement showed no similarities to any previously characterized enzymes (Henriksen et al., 2001).

GAPR-1 is a mammalian protein which is well conserved within vertebrates and known under various aliases such as GLIPR-2 and c9orf19 (Baxter et al., 2006; Eisenberg et al., 2002). In mice, GAPR-1 is predominantly expressed in the lung, spleen, monocytes, uterus and embryonic tissue (Eberle et al., 2002). In humans, it was found in peripheral leukocytes and lung (Eisenberg et al., 2002). Its expression in immunocompetent cells and tissues suggests a role in immunity. Furthermore, it was speculated that GAPR-1 may be involved in the differentiation of epithelial cells into mesenchymal cells, as GAPR-1 was found to be up-regulated in fibrotic human kidneys (Baxter et al., 2006). The molecular function of GAPR-1, however, still remains unclear.

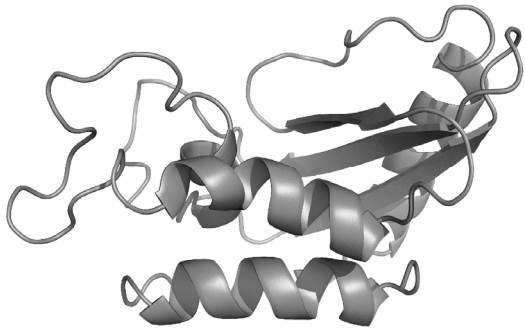
GAPR-1 contains a sperm-coating protein (SCP) domain (13 kDa), which encompasses almost the entire protein (Li et al., 2006). There is no specific function assigned to the SCP domain yet, and proteins containing the SCP domain display heterogeneous functions: the human TPX-1 is probably involved in sperm-egg fertilization (Busso et al., 2007), the cone snail Tex31 possesses proteolytic activity (Milne et al., 2003), the snake venom natrin blocks ion channels (Wang et al., 2006; Wang et al., 2005), and the snake venom ablomin blocks smooth muscle contraction (Yamazaki et al., 2002). In humans, GAPR-1 shows most homology to GLIPR-1 (RTVP-1) and CRISP proteins (Kjeldsen et al., 1996; Kratzschmar et al., 1996). Overall, GAPR-1 shares the highest homology with plant Pathogenesis Related-1 (PR-1) proteins and, in addition, members of this protein family are comparable in size, isoelectric point and 3D structure (Serrano et al., 2004; Szyperski et al., 1998) (Fig. 4).

Crosslink studies, yeast two-hybrid and gel-filtration experiments have shown that GAPR-1 can form dimers (Eberle et al., 2002; Serrano et al., 2004). By formation of a dimer, conserved residues on opposing monomers can form a putative catalytic triad (Serrano et al., 2004). Therefore it was proposed that dimerization is a regulatory mechanism of GAPR-1 function (Serrano et al., 2004).

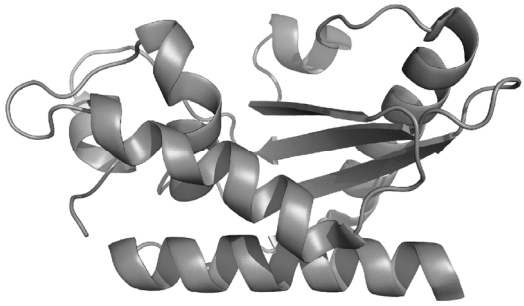
2.2 Membrane binding of GAPR-1 is determined by multiple factors

The membrane binding of GAPR-1 is very strong as salt-stripping of membranes or treatment of cells with Brefeldin A, which causes a redistribution of GAPR-1 in cells, do not release GAPR-1 from membranes. GAPR-1 is myristoylated and this fatty acid modification could provide a mechanism to anchor this protein to the membrane.

P14a



GAPR-1



Ves v 5

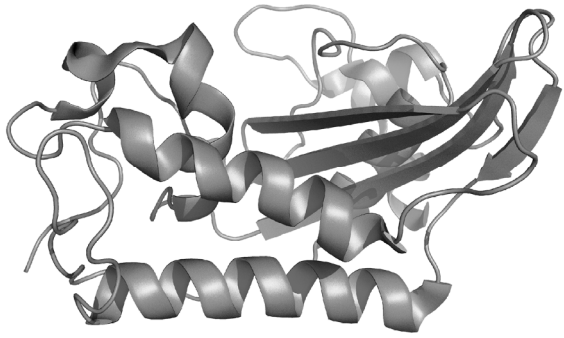


Figure 4.
Comparison of the three-dimensional structure of GAPR-1, p14a (PDB entry 1CFE) and Ves v 5 (PDB entry 1QNX), members of the plant pathogenesis-related group 1 (PR-1). The structure of P14a tomato (upper panel) (Fernandez et al., 1997) is closely related to the structure of GAPR-1 (center) (Serrano et al., 2004) and Ves V 5 (lower panel) (Henriksen et al., 2001). Figures were prepared using pymol software (W. Delano; http://www/pymol.org/).

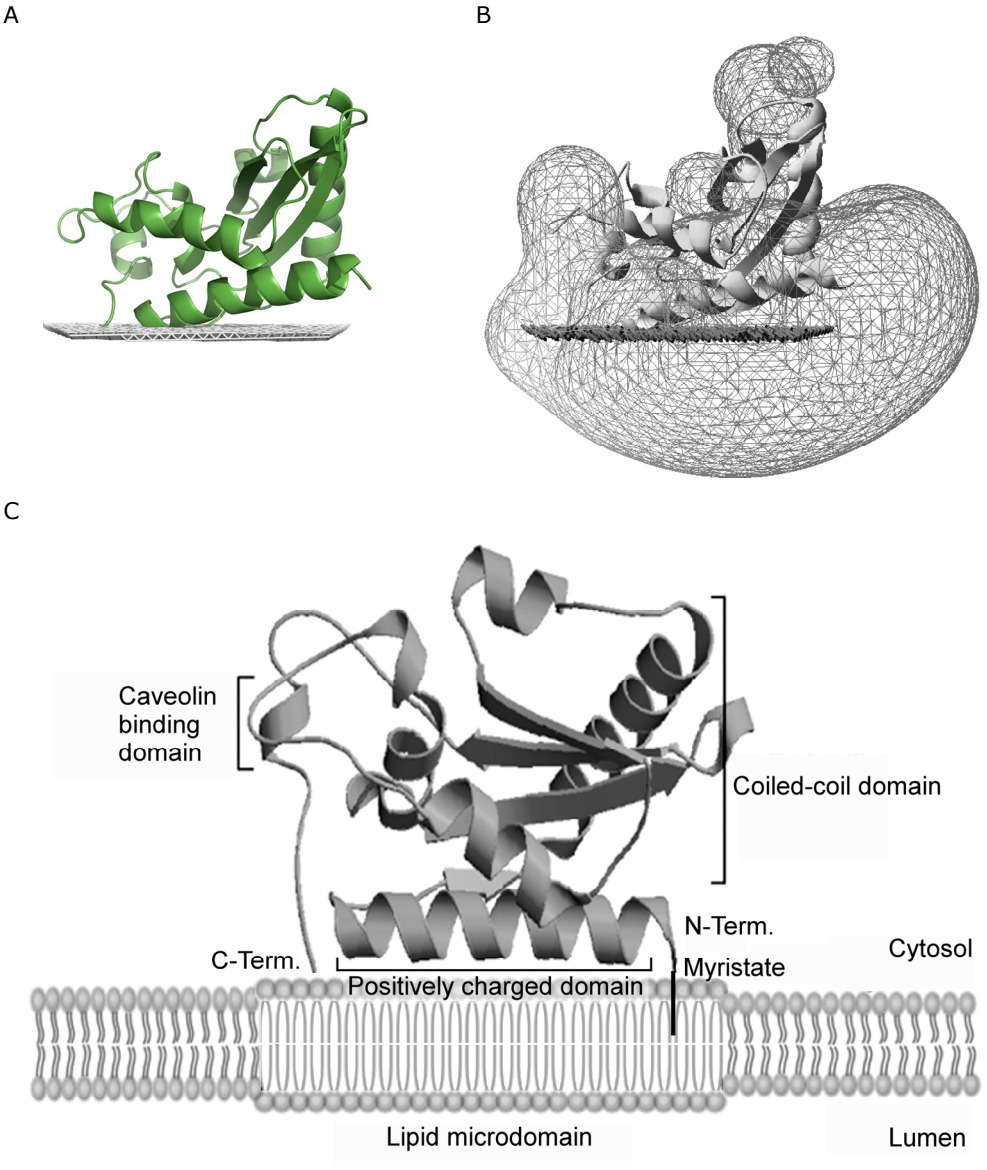


Figure 5. Model of the orientation of GAPR-1 on membranes.
(A) Membrane binding orientation of GAPR-1 as predicted by MAPAS. Structure was visualized by Pymol software. The pdb file can be retrieved from http://cancer-tools.sdsc.edu/R/r_15803.html (B) Same model as in A, but here the positive electric potential is visualized by Swiss PDB viewer (Guex and Peitsch, 1997). (C) Model of GAPR-1 to a membrane. Figure adapted from the thesis of R.L. Serrano, 2003.

The binding energy of myristate is however not sufficient to stably anchor a protein to a membrane (Peitzsch and McLaughlin, 1993). In support of this, several myristoylated proteins do not show exclusive membrane localization and a second interaction is required for efficient membrane-binding [reviewed in (Resh, 1999; Taniguchi, 1999)]. GAPR-1 has been shown to interact with caveolin-1, a well known membrane-resident protein (Eberle et al., 2002). GAPR-1 also has a pI of 9.4, resulting in a net positive charge at physiological pH. According to the crystal structure of GAPR-1, most of the positive charges are localized to one area of the protein surface (Fig. 5B) (Serrano et al., 2004), creating an opportunity for efficient electrostatic interactions with negatively charged lipids in the membrane. Electrostatic lipid and protein interactions, combined with myristoyl anchorage may be sufficient for stable membrane binding (Resh, 1999). Based on the structural features of GAPR-1, such as the localization of the positive charges on the molecular surface of GAPR-1, the N-terminal myristate group, the caveolin binding motif, and the coiled-coil region, we proposed a membrane binding orientation of GAPR-1 (Fig. 5C). A computer model, MAPAS, that was recently published and that can predict membrane-protein interactions, confirmed our previous membrane-binding model (Fig. 5A) (Sharikov et al., 2008). The MAPAS model predicts that the electrostatic potential of non-myristoylated GAPR-1 is not fully sufficient for stable membrane binding. The two-signal model as proposed by M.Resh (Resh, 1999) can explain why GAPR-1 is stably localized to the membrane. This model predicts that in addition to myristoylation, a second interaction is required for stable membrane anchorage. In addition to myristoylation, GAPR-1 has a positively charged protein surface, an interaction with the membrane resident protein caveolin-1, and a coiled-coil domain for additional protein-protein interactions (Eberle et al., 2002). The above mentioned characteristics could explain the stable membrane localization of GAPR-1 according to the two signal model.

Outline of the thesis

GAPR-1 is a membrane bound PR-1 family member that is localized to the Golgi complex, but may be relocated to other cellular organelles, such as phagosomes or exosomes. The aim of the research described in this thesis was to characterize the interactions of GAPR-1 with biological and model membranes.

The membrane binding characteristics of GAPR-1 are addressed in chapter 2, which describes how GAPR-1 interacts with membranes containing monovalent negatively charged lipids. The nature of a very stable interaction of GAPR-1 with phosphatidylinositol (PI) is investigated using multiple independent methods. How the interaction of GAPR-1 with PI may be regulated is described in Chapter 3. The interaction of GAPR-1 with phytic acid, an inositol phosphate, is investigated by 3D structure analysis and gel-filtration chromatography. Based on the obtained structural model, mutants were designed and tested for their prediction to interfere with the novel structure. In Chapter 4 the affinity of GAPR-1 for phosphoinositides was investigated by two separate approaches. In addition, the contribution of myristoylation of GAPR-1 to membrane affinity and raft participation was investigated by mutagenesis. In Chapter 5 the findings of chapters 2-4 are summarized and discussed.

References

Abrami, L., M. Fivaz, P.E. Glauser, R.G. Parton, and F.G. van der Goot. 1998. A pore-forming toxin interacts with a GPI-anchored protein and causes vacuolation of the endoplasmic reticulum. *J Cell Biol*. 140:525-40.

Adachi, J., C. Kumar, Y. Zhang, J.V. Olsen, and M. Mann. 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biol*. 7:R80.

Ahmed, S.N., D.A. Brown, and E. London. 1997. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry*. 36:10944-53.

Alexander, D., R.M. Goodman, M. Gut-Rella, C. Glascock, K. Weymann, L. Friedrich, D. Maddox, P. Ahl-Goy, T. Luntz, E. Ward, and et al. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc Natl Acad Sci U S A*. 90:7327-31.

Allen, L.A., J.A. Allgood, X. Han, and L.M. Wittine. 2005. Phosphoinositide3-kinase regulates actin polymerization during delayed phagocytosis of Helicobacter pylori. *J Leukoc Biol*. 78:220-30.

Almeida, P.F., W.L. Vaz, and T.E. Thompson. 1993. Percolation and diffusion in three-component lipid bilayers: effect of cholesterol on an equimolar mixture of two phosphatidylcholines. *Biophys J*. 64:399-412.

Anderson, R.G. 1998. The caveolae membrane system. *Annu Rev Biochem*. 67:199-225.

Arellano-Reynoso, B., N. Lapaque, S. Salcedo, G. Briones, A.E. Ciocchini, R. Ugalde, E. Moreno, I. Moriyon, and J.P. Gorvel. 2005. Cyclic beta-1,2-glucan is a Brucella virulence factor required for intracellular survival. *Nat Immunol*. 6:618-25.

Bagnat, M., S. Keranen, A. Shevchenko, and K. Simons. 2000. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc Natl Acad Sci U S A*. 97:3254-9.

Bankaitis, V.A., J.R. Aitken, A.E. Cleves, and W. Dowhan. 1990. An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature*. 347:561-2.

Baxter, R.M., T.P. Crowell, J.A. George, M.E. Getman, and H. Gardner. 2006. The plant pathogenesis related protein GLIPR-2 is highly expressed in fibrotic kidney and promotes epithelial to mesenchymal transition in vitro. *Matrix Biol*.

Berridge, M.J., and R.F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature*. 341:197-205.

Bickel, P.E., P.E. Scherer, J.E. Schnitzer, P. Oh, M.P. Lisanti, and H.F. Lodish. 1997. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *J Biol Chem*. 272:13793-802.

Boggs, J.M. 1987. Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function. *Biochim Biophys Acta*. 906:353-404.

Bretscher, M.S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science*. 261:1280-1.

Brown, D.A. 2006. Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology (Bethesda)*. 21:430-9.

Brown, D.A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol*. 14:111-136.

Brugger, B., B. Glass, P. Haberkant, I. Leibrecht, F.T. Wieland, and H.G. Krausslich. 2006. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci U S A*. 103:2641-6.

Burlak, C., A.R. Whitney, D.J. Mead, T. Hackstadt, and F.R. Deleo. 2006. Maturation of human neutrophil phagosomes includes incorporation of molecular chaperones and endoplasmic reticulum quality control machinery. *Mol Cell Proteomics*. 5:620-34.

Busso, D., N.M. Goldweic, M. Hayashi, M. Kasahara, and P.S. Cuasnicu. 2007. Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. *Biol Reprod*. 76:701-8.

Celli, J., M. Olivier, and B.B. Finlay. 2001. Enteropathogenic Escherichia coli mediates antiphagocytosis through the inhibition of PI 3-kinase-dependent pathways. *Embo J*. 20:1245-58.

Charron, A.J., and L.D. Sibley. 2002. Host cells: mobilizable lipid resources for the intracellular parasite Toxoplasma gondii. *J Cell Sci*. 115:3049-59.

Chua, J., and V. Deretic. 2004. Mycobacterium tuberculosis reprograms waves of phosphatidylinositol 3-phosphate on phagosomal organelles. *J Biol Chem*. 279:36982-36992.

Cluett, E.B., and C.E. Machamer. 1996. The envelope of vaccinia virus reveals an unusual phospholipid in Golgi complex membranes. *J Cell Sci*. 109 (Pt 8):2121-31.

Coppens, I., and K.A. Joiner. 2003. Host but not parasite cholesterol controls Toxoplasma cell entry by modulating organelle discharge. *Mol Biol Cell*. 14:3804-20.
Coppolino, M.G., R. Dierckman, J. Loijens, R.F. Collins, M. Pouladi, J. Jongstra-Bilen, A.D. Schreiber, W.S. Trimble, R. Anderson, and S. Grinstein. 2002. Inhibition of phosphatidylinositol-4-phosphate 5-kinase Ialpha impairs localized actin remodeling and suppresses phagocytosis. *J Biol Chem*. 277:43849-57.
Damm, E.M., L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, and A. Helenius. 2005. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell Biol*. 168:477-88.
Dangl, J.L., and J.D. Jones. 2001. Plant pathogens and integrated defence responses to infection. *Nature*. 411:826-33.
De Matteis, M.A., and A. Godi. 2004. PI-loting membrane traffic. *Nat Cell Biol*. 6:487-92.
Dermine, J.F., S. Duclos, J. Garin, F. St-Louis, S. Rea, R.G. Parton, and M. Desjardins. 2001. Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. *J Biol Chem*. 276:18507-12.
Dermine, J.F., G. Goyette, M. Houde, S.J. Turco, and M. Desjardins. 2005. Leishmania donovani lipophosphoglycan disrupts phagosome microdomains in J774 macrophages. *Cell Microbiol*. 7:1263-70.
Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature*. 443:651-7.
Dickson, R.C. 1998. Sphingolipid functions in Saccharomyces cerevisiae: comparison to mammals. *Annu Rev Biochem*. 67:27-48.
Drab, M., P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindschau, F. Mende, F.C. Luft, A. Schedl, H. Haller, and T.V. Kurzchalia. 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science*. 293:2449-52.
Eberle, H.B., R.L. Serrano, J. Fullekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, and J.B. Helms. 2002. Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex. *J Cell Sci*. 115:827-838.
Eidels, L., R.L. Proia, and D.A. Hart. 1983. Membrane receptors for bacterial toxins. *Microbiol Rev*. 47:596-620.
Eisenberg, I., M. Barash, T. Kahan, and S. Mitrani-Rosenbaum. 2002. Cloning and characterization of a human novel gene C9orf19 encoding a conserved putative protein with an SCP-like extracellular protein domain. *Gene*. 293:141-148.
Eisenkolb, M., C. Zenzmaier, E. Leitner, and R. Schneiter. 2002. A specific structural requirement for ergosterol in long-chain fatty acid synthesis mutants important for maintaining raft domains in yeast. *Mol Biol Cell*. 13:4414-28.
Fahy, E., S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, Jr., R.C. Murphy, C.R. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, and E.A. Dennis. 2005. A comprehensive classification system for lipids. *J Lipid Res*. 46:839-61.
Fernandez, C., T. Szyperski, T. Bruyere, P. Ramage, E. Mosinger, and K. Wuthrich. 1997. NMR solution structure of the pathogenesis-related protein P14a. *J Mol Biol*. 266:576-93.
Fivaz, M., L. Abrami, and F.G. van der Goot. 1999. Landing on lipid rafts. *Trends Cell Biol*. 9:212-3.
Fratti, R.A., J. Chua, I. Vergne, and V. Deretic. 2003. Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc Natl Acad Sci U S A*. 100:5437-5442.
Futerman, A.H., B. Stieger, A.L. Hubbard, and R.E. Pagano. 1990. Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus. *J Biol Chem*. 265:8650-7.
Gally, H.U., A. Seelig, and J. Seelig. 1976. Cholesterol-induced rod-like motion of fatty acyl chains in lipid bilayers a deuterium magnetic resonance study. *Hoppe Seylers Z Physiol Chem*. 357:1447-50.
Garin, J., R. Diez, S. Kieffer, J.F. Dermine, S. Duclos, E. Gagnon, R. Sadoul, C. Rondeau, and M. Desjardins. 2001. The phagosome proteome: insight into phagosome functions. *J Cell Biol*. 152:165-80.

Gassama-Diagne, A., W. Yu, M. ter Beest, F. Martin-Belmonte, A. Kierbel, J. Engel, and K. Mostov. 2006. Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nat Cell Biol*. 8:963-70.

Gekara, N.O., T. Jacobs, T. Chakraborty, and S. Weiss. 2005. The cholesterol-dependent cytolysin listeriolysin O aggregates rafts via oligomerization. *Cell Microbiol*. 7:1345-56.

Gkantiragas, I., B. Brugger, E. Stuven, D. Kaloyanova, X.Y. Li, K. Lohr, F. Lottspeich, F.T. Wieland, and J.B. Helms. 2001. Sphingomyelin-enriched microdomains at the Golgi complex. *Mol Biol Cell*. 12:1819-1833.

Glebov, O.O., and B.J. Nichols. 2004. Lipid raft proteins have a random distribution during localized activation of the T-cell receptor. *Nat Cell Biol*. 6:238-43.

Goligorsky, M.S., H. Li, S. Brodsky, and J. Chen. 2002. Relationships between caveolae and eNOS: everything in proximity and the proximity of everything. *Am J Physiol Renal Physiol*. 283:F1-10.

Grimmer, S., T.G. Iversen, B. van Deurs, and K. Sandvig. 2000. Endosome to Golgi transport of ricin is regulated by cholesterol. *Mol Biol Cell*. 11:4205-16.

Gruenberg, J., and F.G. van der Goot. 2006. Mechanisms of pathogen entry through the endosomal compartments. *Nat Rev Mol Cell Biol*. 7:495-504.

Guex, N., and M.C. Peitsch. 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 18:2714-23.

Hammond-Kosack, K.E., and J.D. Jones. 1996. Resistance gene-dependent plant defense responses. *Plant Cell*. 8:1773-91.

Hannun, Y.A., and L.M. Obeid. 2008. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol*. 9:139-50.

Hansen, G.H., L.L. Niels-Christiansen, E. Thorsen, L. Immerdal, and E.M. Danielsen. 2000. Cholesterol depletion of enterocytes. Effect on the Golgi complex and apical membrane trafficking. *J Biol Chem*. 275:5136-42.

Harder, T., P. Scheiffele, P. Verkade, and K. Simons. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol*. 141:929-42.

Haucke, V., and G. Di Paolo. 2007. Lipids and lipid modifications in the regulation of membrane traffic. *Curr Opin Cell Biol*. 19:426-35.

Heerklotz, H. 2002. Triton promotes domain formation in lipid raft mixtures. *Biophys J*. 83:2693-701.

Heil, M., and R.M. Bostock. 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Ann Bot (Lond)*. 89:503-12.

Helms, J.B., D. Helms-Brons, B. Brugger, I. Gkantiragas, H. Eberle, W. Nickel, B. Nurnberg, H.H. Gerdes, and F.T. Wieland. 1998. A putative heterotrimeric G protein inhibits the fusion of COPI-coated vesicles. Segregation of heterotrimeric G proteins from COPI-coated vesicles. *J Biol Chem*. 273:15203-8.

Helms, J.B., and C. Zurzolo. 2004. Lipids as targeting signals: lipid rafts and intracellular trafficking. *Traffic*. 5:247-254.

Henriksen, A., T.P. King, O. Mirza, R.I. Monsalve, K. Meno, H. Ipsen, J.N. Larsen, M. Gajhede, and M.D. Spangfort. 2001. Major venom allergen of yellow jackets, Ves v 5: structural characterization of a pathogenesis-related protein superfamily. *Proteins*. 45:438-48.

Hernandez, L.D., K. Hueffer, M.R. Wenk, and J.E. Galan. 2004. Salmonella modulates vesicular traffic by altering phosphoinositide metabolism. *Science*. 304:1805-1807.

Hess, S.T., M. Kumar, A. Verma, J. Farrington, A. Kenworthy, and J. Zimmerberg. 2005. Quantitative electron microscopy and fluorescence spectroscopy of the membrane distribution of influenza hemagglutinin. *J Cell Biol*. 169:965-76.

Hilbi, H. 2006. Modulation of phosphoinositide metabolism by pathogenic bacteria. *Cell Microbiol*.

Hullin-Matsuda, F., and T. Kobayashi. 2007. Monitoring the distribution and dynamics of signaling microdomains in living cells with lipid-specific probes. *Cell Mol Life Sci*. 64:2492-504.

Ikonen, E. 2001. Roles of lipid rafts in membrane transport. *Curr Opin Cell Biol*. 13:470-477.

Ikonomov, O.C., D. Sbrissa, M. Foti, J.L. Carpentier, and A. Shisheva. 2003. PIKfyve controls fluid phase endocytosis but not recycling/degradation of endocytosed receptors or sorting of procathepsin D by regulating multivesicular body morphogenesis. *Mol Biol Cell*. 14:4581-91.

Ipsen, J.H., G. Karlstrom, O.G. Mouritsen, H. Wennerstrom, and M.J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim Biophys Acta*. 905:162-72.

Jacobson, K., O.G. Mouritsen, and R.G. Anderson. 2007. Lipid rafts: at a crossroad between cell biology and physics. *Nat Cell Biol*. 9:7-14.

Jeckel, D., A. Karrenbauer, K.N. Burger, G. van Meer, and F. Wieland. 1992. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J Cell Biol*. 117:259-67.
Kapadia, S.B., and F.V. Chisari. 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci U S A*. 102:2561-6.
Kierbel, A., A. Gassama-Diagne, K. Mostov, and J.N. Engel. 2005. The phosphoinositol-3-kinase-protein kinase B/Akt pathway is critical for Pseudomonas aeruginosa strain PAK internalization. *Mol Biol Cell*. 16:2577-85.
Kierbel, A., A. Gassama-Diagne, C. Rocha, L. Radoshevich, J. Olson, K. Mostov, and J. Engel. 2007. Pseudomonas aeruginosa exploits a PIP3-dependent pathway to transform apical into basolateral membrane. *J Cell Biol*. 177:21-7.
King, C.A., and W.E. Van Heyningen. 1973. Deactivation of cholera toxin by a sialidase-resistant monosialosylganglioside. *J Infect Dis*. 127:639-47.
Kiyokawa, E., T. Baba, N. Otsuka, A. Makino, S. Ohno, and T. Kobayashi. 2005. Spatial and functional heterogeneity of sphingolipid-rich membrane domains. *J Biol Chem*. 280:24072-84.
Kjeldsen, L., J.B. Cowland, A.H. Johnsen, and N. Borregaard. 1996. SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. *FEBS Lett*. 380:246-50.
Kratzschmar, J., B. Haendler, U. Eberspaecher, D. Roosterman, P. Donner, and W.D. Schleuning. 1996. The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. *Eur J Biochem*. 236:827-36.
Krauss, K., and P. Altevogt. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *J Biol Chem*. 274:36921-7.
Lacalle, R.A., E. Mira, C. Gomez-Mouton, S. Jimenez-Baranda, A.C. Martinez, and S. Manes. 2002. Specific SHP-2 partitioning in raft domains triggers integrin-mediated signaling via Rho activation. *J Cell Biol*. 157:277-89.
Lamb, C., and R.A. Dixon. 1997. The Oxidative Burst in Plant Disease Resistance. *Annu Rev Plant Physiol Plant Mol Biol*. 48:251-275.
Legesse-Miller, A., Y. Sagiv, R. Glozman, and Z. Elazar. 2000. Aut7p, a soluble autophagic factor, participates in multiple membrane trafficking processes. *J Biol Chem*. 275:32966-73.
Lemmon, M.A. 2008. Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol*. 9:99-111.
Li, S., Y. Shin, K.W. Cho, and C.S. Merzdorf. 2006. The Xfeb gene is directly upregulated by Zic1 during early neural development. *Dev Dyn*. 235:2817-27.
Li, X., D. Kaloyanova, M. van Eijk, R. Eerland, G. van der Goot, V. Oorschot, J. Klumperman, F. Lottspeich, V. Starkuviene, F.T. Wieland, and J.B. Helms. 2007. Involvement of a Golgi-resident GPI-anchored protein in maintenance of the Golgi structure. *Mol Biol Cell*. 18:1261-71.
Lichtenberg, D., F.M. Goni, and H. Heerklotz. 2005. Detergent-resistant membranes should not be identified with membrane rafts. *Trends Biochem Sci*. 30:430-6.
Lisanti, M.P., and E. Rodriguez-Boulan. 1990. Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. *Trends Biochem Sci*. 15:113-8.
Lisanti, M.P., Z. Tang, P.E. Scherer, E. Kubler, A.J. Koleske, and M. Sargiacomo. 1995. Caveolae, transmembrane signalling and cellular transformation. *Mol Membr Biol*. 12:121-4.
Litvak, V., Y.D. Shaul, M. Shulewitz, R. Amarilio, S. Carmon, and S. Lev. 2002. Targeting of Nir2 to lipid droplets is regulated by a specific threonine residue within its PI-transfer domain. *Curr Biol*. 12:1513-8.
Madore, N., K.L. Smith, C.H. Graham, A. Jen, K. Brady, S. Hall, and R. Morris. 1999. Functionally different GPI proteins are organized in different domains on the neuronal surface. *Embo J*. 18:6917-26.
Mallard, F., C. Antony, D. Tenza, J. Salamero, B. Goud, and L. Johannes. 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. *J Cell Biol*. 143:973-90.
Manes, S., E. Mira, C. Gomez-Mouton, R.A. Lacalle, P. Keller, J.P. Labrador, and A.C. Martinez. 1999. Membrane raft microdomains mediate front-rear polarity in migrating cells. *Embo J*. 18:6211-20.
Mazumdar, J., and B. Striepen. 2007. Make it or take it: fatty acid metabolism of apicomplexan parasites. *Eukaryot Cell*. 6:1727-35.
Merritt, E.A., S. Sarfaty, F. van den Akker, C. L'Hoir, J.A. Martial, and W.G. Hol. 1994. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci*. 3:166-75.

Milne, T.J., G. Abbenante, J.D. Tyndall, J. Halliday, and R.J. Lewis. 2003. Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. *J Biol Chem*. 278:31105-31110.
Monier, S., R.G. Parton, F. Vogel, J. Behlke, A. Henske, and T.V. Kurzchalia. 1995. VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. *Mol Biol Cell*. 6:911-27.
Mukherjee, S., and F.R. Maxfield. 2000. Role of membrane organization and membrane domains in endocytic lipid trafficking. *Traffic*. 1:203-11.
Munoz-Elias, E.J., and J.D. McKinney. 2005. Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med*. 11:638-44.
Munro, S. 2003. Lipid rafts: elusive or illusive? *Cell*. 115:377-388.
Nguyen, D.H., and J.E. Hildreth. 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J Virol*. 74:3264-72.
Nichols, B.J., A.K. Kenworthy, R.S. Polishchuk, R. Lodge, T.H. Roberts, K. Hirschberg, R.D. Phair, and J. Lippincott-Schwartz. 2001. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J Cell Biol*. 153:529-41.
Niderman, T., I. Genetet, T. Bruyere, R. Gees, A. Stintzi, M. Legrand, B. Fritig, and E. Mosinger. 1995. Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against Phytophthora infestans. *Plant Physiol*. 108:17-27.
Niebuhr, K., S. Giuriato, T. Pedron, D.J. Philpott, F. Gaits, J. Sable, M.P. Sheetz, C. Parsot, P.J. Sansonetti, and B. Payrastre. 2002. Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the S.flexneri effector IpgD reorganizes host cell morphology. *Embo J*. 21:5069-78.
Nurnberger, T., F. Brunner, B. Kemmerling, and L. Piater. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev*. 198:249-66.
Orlandi, P.A., and P.H. Fishman. 1998. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biol*. 141:905-15.
Pagano, R.E., V. Puri, M. Dominguez, and D.L. Marks. 2000. Membrane traffic in sphingolipid storage diseases. *Traffic*. 1:807-15.
Palestini, P., M. Allietta, S. Sonnino, G. Tettamanti, T.E. Thompson, and T.W. Tillack. 1995. Gel phase preference of ganglioside GM1 at low concentration in two-component, two-phase phosphatidylcholine bilayers depends upon the ceramide moiety. *Biochim Biophys Acta*. 1235:221-30.
Peitzsch, R.M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry*. 32:10436-43.
Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol*. 3:473-83.
Pierini, L.M., and F.R. Maxfield. 2001. Flotillas of lipid rafts fore and aft. *Proc Natl Acad Sci U S A*. 98:9471-3.
Pisitkun, T., R.F. Shen, and M.A. Knepper. 2004. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 101:13368-73.
Prior, I.A., C. Muncke, R.G. Parton, and J.F. Hancock. 2003. Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J Cell Biol*. 160:165-70.
Qualmann, B., and M.M. Kessels. 2002. Endocytosis and the cytoskeleton. *Int Rev Cytol*. 220:93-144.
Quitard, S., P. Dean, M. Maresca, and B. Kenny. 2006. The enteropathogenic Escherichia coli EspF effector molecule inhibits PI-3 kinase-mediated uptake independently of mitochondrial targeting. *Cell Microbiol*. 8:972-81.
Reinhart, M.P., J.T. Billheimer, J.R. Faust, and J.L. Gaylor. 1987. Subcellular localization of the enzymes of cholesterol biosynthesis and metabolism in rat liver. *J Biol Chem*. 262:9649-55.
Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta*. 1451:1-16.
Rog, T., and M. Pasenkiewicz-Gierula. 2001. Cholesterol effects on the phospholipid condensation and packing in the bilayer: a molecular simulation study. *FEBS Lett*. 502:68-71.
Rogers, L.D., and L.J. Foster. 2007. The dynamic phagosomal proteome and the contribution of the endoplasmic reticulum. *Proc Natl Acad Sci U S A*. 104:18520-5.
Rosenberger, C.M., and B.B. Finlay. 2003. Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. *Nat Rev Mol Cell Biol*. 4:385-96.
Sankaram, M.B., and T.E. Thompson. 1990. Interaction of cholesterol with various glycerophospholipids and sphingomyelin. *Biochemistry*. 29:10670-5.

Schuck, S., M. Honsho, K. Ekroos, A. Shevchenko, and K. Simons. 2003. Resistance of cell membranes to different detergents. *Proc Natl Acad Sci U S A*. 100:5795-5800.
Schutz, G.J., G. Kada, V.P. Pastushenko, and H. Schindler. 2000. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. *Embo J*. 19:892-901.
Serrano, R.L., A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, and M.R. Groves. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. *J Mol Biol*. 339:173-83.
Sharikov, Y., R.C. Walker, J. Greenberg, V. Kouznetsova, S.K. Nigam, M.A. Miller, E. Masliah, and I.F. Tsigelny. 2008. MAPAS: a tool for predicting membrane-contacting protein surfaces. *Nat Methods*. 5:119.
Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature*. 387:569-572.
Simons, K., and W.L. Vaz. 2004. Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct*. 33:269-95.
Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science*. 175:720-31.
Stoorvogel, W., M.J. Kleijmeer, H.J. Geuze, and G. Raposo. 2002. The biogenesis and functions of exosomes. *Traffic*. 3:321-330.
Stueven, E., A. Porat, F. Shimron, E. Fass, D. Kaloyanova, B. Bruegger, F.T. Wieland, Z. Elazar, and J.B. Helms. 2003. Intra-Golgi protein transport depends on a cholesterol balance in the lipid membrane. *J Biol Chem*.
Su, A.I., J.P. Pezacki, L. Wodicka, A.D. Brideau, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R.H. Purcell, P.G. Schultz, and F.V. Chisari. 2002. Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci U S A*. 99:15669-74.
Sud, M., E. Fahy, D. Cotter, A. Brown, E.A. Dennis, C.K. Glass, A.H. Merrill, Jr., R.C. Murphy, C.R. Raetz, D.W. Russell, and S. Subramaniam. 2007. LMSD: LIPID MAPS structure database. *Nucleic Acids Res*. 35:D527-32.
Szyperski, T., C. Fernandez, C. Mumenthaler, and K. Wuthrich. 1998. Structure comparison of human glioma pathogenesis-related protein GliPR and the plant pathogenesis-related protein P14a indicates a functional link between the human immune system and a plant defense system. *Proc Natl Acad Sci U S A*. 95:2262-2266.
Takeda, M., G.P. Leser, C.J. Russell, and R.A. Lamb. 2003. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc Natl Acad Sci U S A*. 100:14610-7.
Taniguchi, H. 1999. Protein myristoylation in protein-lipid and protein-protein interactions. *Biophys Chem*. 82:129-37.
Terebiznik, M.R., O.V. Vieira, S.L. Marcus, A. Slade, C.M. Yip, W.S. Trimble, T. Meyer, B.B. Finlay, and S. Grinstein. 2002. Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by Salmonella. *Nat Cell Biol*. 4:766-73.
Tweten, R.K. 2005. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. *Infect Immun*. 73:6199-209.
van der Goot, F.G., and T. Harder. 2001. Raft membrane domains: from a liquid-ordered membrane phase to a site of pathogen attack. *Semin Immunol*. 13:89-97.
van Loon, L.C., M. Rep, and C.M. Pieterse. 2006. Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol*. 44:135-62.
van Loon, L.C., and A. van Kammen. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var. "Samsun" and "Samsun NN". II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology*. 40:190-211.
van Loon, L.C., and E.A. van Strien. 1999. The families of plant pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol*. 55:85-97.
van Meer, G. 2005. Cellular lipidomics. *Embo J*. 24:3159-65.
van Meer, G., and K. Simons. 1982. Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cells have unique phospholipid compositions. *Embo J*. 1:847-52.
van Rheenen, J., E.M. Achame, H. Janssen, J. Calafat, and K. Jalink. 2005. PIP2 signaling in lipid domains: a critical re-evaluation. *Embo J*. 24:1664-73.
Vergne, I., J. Chua, H.H. Lee, M. Lucas, J. Belisle, and V. Deretic. 2005. Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A*. 102:4033-4038.
Vergne, I., R.A. Fratti, P.J. Hill, J. Chua, J. Belisle, and V. Deretic. 2004. Mycobacterium tuberculosis phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. *Mol Biol Cell*. 15:751-60.

Volonte, D., F. Galbiati, S. Li, K. Nishiyama, T. Okamoto, and M.P. Lisanti. 1999. Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. *J Biol Chem*. 274:12702-9.

Wang, F., H. Li, M.N. Liu, H. Song, H.M. Han, Q.L. Wang, C.C. Yin, Y.C. Zhou, Z. Qi, Y.Y. Shu, Z.J. Lin, and T. Jiang. 2006. Structural and functional analysis of natrin, a venom protein that targets various ion channels. *Biochem Biophys Res Commun*. 351:443-8.

Wang, J., B. Shen, M. Guo, X. Lou, Y. Duan, X.P. Cheng, M. Teng, L. Niu, Q. Liu, Q. Huang, and Q. Hao. 2005. Blocking effect and crystal structure of natrin toxin, a cysteine-rich secretory protein from Naja atra venom that targets the BKCa channel. *Biochemistry*. 44:10145-52.

Wolf, A.A., M.G. Jobling, S. Wimer-Mackin, M. Ferguson-Maltzman, J.L. Madara, R.K. Holmes, and W.I. Lencer. 1998. Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J Cell Biol*. 141:917-27.

Yamazaki, Y., H. Koike, Y. Sugiyama, K. Motoyoshi, T. Wada, S. Hishinuma, M. Mita, and T. Morita. 2002. Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. *Eur J Biochem*. 269:2708-15.

Yeung, T., B. Ozdamar, P. Paroutis, and S. Grinstein. 2006. Lipid metabolism and dynamics during phagocytosis. *Curr Opin Cell Biol*. 18:429-37.

Yoshizaki, F., H. Nakayama, C. Iwahara, K. Takamori, H. Ogawa, and K. Iwabuchi. 2007. Role of glycosphingolipid-enriched microdomains in innate immunity: Microdomain-dependent phagocytic cell functions. *Biochim Biophys Acta*.

Zhou, D., L.M. Chen, L. Hernandez, S.B. Shears, and J.E. Galan. 2001. A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol Microbiol*. 39:248-59.

Chapter 2

Strong Binding of GAPR-1 to Phosphatidylinositol: Indications for a Novel Membrane Anchor

Josse van Galen*, Bas van Balkom[$], Ernstpeter Stüven*, J. Bernd Helms*

*Department of Biochemistry and Cell Biology and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands

[$]Institute for Veterinary Research, IVW/GSAH, Utrecht, The Netherlands; Department of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Submitted

Abstract

Golgi-Associated Plant Pathogenesis-Related protein 1 (GAPR-1) is a mammalian protein that belongs to the superfamily of plant pathogenesis related proteins group 1 (PR-1). GAPR-1 strongly associates with the cytosolic leaflet of the Golgi membrane and it is enriched in lipid rafts. The myristoyl moiety at the N-terminus of GAPR-1 is not sufficient for stable membrane anchorage and other protein or lipid interactions are likely to be involved. By use of a liposome binding assay, we investigated the interaction of GAPR-1 with lipids. Here we report that non-myristoylated GAPR-1 binds to negatively charged lipids with highest preference for phosphatidic acid. Interestingly, GAPR-1 binds to phosphatidylinositol with unusual characteristics. Denaturation or organic extraction of GAPR-1 does not result in dissociation of phosphatidylinositol from GAPR-1. Mass spectrometric analysis showed that up to 3 molecules of phosphatidylinositol can bind to GAPR-1. Our data indicate that GAPR-1 may bind covalently to phosphatidylinositol. The implications of this novel membrane anchor on the membrane binding characteristics and function of GAPR-1 are discussed.

Introduction

Golgi-Associated Plant Pathogenesis-Related protein 1 (GAPR-1) is a mammalian protein which is well conserved among vertebrates and known under various aliases such as GLIPR-2 and c9orf19 (Baxter et al., 2007; Eisenberg et al., 2002). In mice, GAPR-1 is predominantly expressed in the lung, spleen, monocytes, uterus and embryonic tissue (Eberle et al., 2002). In humans, GAPR-1 expression has thus far been described in peripheral leukocytes and lung (Eisenberg et al., 2002). Its expression in immunocompetent cells and tissues suggests a role in immunity. Furthermore, it was speculated that GAPR-1 may be involved in the differentiation of epithelial cells into mesenchymal cells as GAPR-1 was found to be upregulated in fibrotic human kidneys (Baxter et al., 2007). The biological function of GAPR-1 remains, however, unclear.

Structural analysis *in silico* has shown that GAPR-1 contains a sperm-coating protein (SCP) domain (13 kDa), which encompasses almost the entire protein (17 kDa) (Li et al., 2006). So far, no specific function has been assigned to the SCP domain and proteins containing the SCP domain display heterogeneous functions (Busso et al., 2007; Milne et al., 2003; Wang et al., 2006; Wang et al., 2005; Yamazaki et al., 2002). In humans, GAPR-1 shows significant homology to GLIPR-1 (RTVP-1) and CRISP proteins (Kjeldsen et al., 1996; Kratzschmar et al., 1996). Overall, GAPR-1 shares the highest homology with plant Pathogenesis Related-1 (PR-1) proteins, which are proteins that have been implicated in acquisition of resistance by plants against pathogens [reviewed in (van Loon and van Strien, 1999)]. Members of this protein family are comparable to GAPR-1 in size, isoelectric point and 3D structure (Szyperski et al., 1998). In contrast to plant PR-1 proteins and other mammalian SCP domain containing proteins, GAPR-1 is not secreted as it lacks a signal peptide. Instead, it localizes to the cytosolic leaflet of lipid rafts at the Golgi complex (Gkantiragas et al., 2001).

The membrane binding is very strong, as salt-stripping of membranes or treatment of cells with Brefeldin A, which causes a redistribution of GAPR-1 in cells, do not release GAPR-1 from membranes. The strong binding to Golgi membranes is unique as most of the proteins containing an SCP domain are localized to the extracellular leaflet of the plasma membrane or remain soluble upon secretion (Busso et al., 2007; Li et al., 2006; Milne et al., 2003; van Loon and van Strien, 1999). GAPR-1 is myristoylated and this fatty acid modification could provide a mechanism to anchor this protein to the membrane. The binding energy of myristate is however

not sufficient to stably anchor a protein to a membrane (Peitzsch and McLaughlin, 1993). In agreement with this, several myristoylated proteins do not show exclusive membrane localization, and a second interaction is required for efficient membrane-binding [reviewed in (Resh, 1999; Taniguchi, 1999)]. GAPR-1 has been shown to interact with caveolin-1, a well known membrane resident protein (Eberle et al., 2002). GAPR-1 also has a pI of 9.4, resulting in a net positive charge at physiological pH. According to the crystal structure of GAPR-1, most of the positive charges are localized to one area of the protein surface (Serrano et al., 2004), creating an opportunity for efficient electrostatic interactions with negatively charged lipids in the membrane. Electrostatic lipid-protein interactions combined with myristoyl anchorage may be sufficient for stable membrane binding (Resh, 1999).

To gain more insight into the membrane-binding mechanism of GAPR-1, we investigated how GAPR-1 interacts with lipids. By use of a liposome binding assay, we show that GAPR-1 binds to negatively charged lipids. The binding to phosphatidylinositol (PI) showed unusual properties that collectively suggest that GAPR-1 binds strongly to PI. Our data suggest a novel mechanism for stable anchorage of peripheral proteins to membranes.

Materials and methods

Lipids

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate, L-a-phosphatidy-linositol (Bovine liver), sphingomyelin (egg), cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] were purchased from Avanti Polar Lipids (Alabaster, U.S.).

Liposome binding assay.

Stock solutions of lipids were made in chloroform/methanol (1:2 v/v) and stored in the freezer at -20 °C under N_2. Liposomes were made freshly for each liposome binding assay. To generate liposomes, phospholipids and cholesterol were mixed from stock solutions in a molar ratio of 2.28:1. The solvent was evaporated using a flow of N_2 with subsequent drying at room temperature in a speedvac (Savant SVC100H, Farmingdale, U.S.) for at least 90 minutes. Subsequently, 50-NT buffer (50 mM NaCl, 25 mM tris, pH 7.4) was added on the dried lipid film to a final

concentration of 6.8 µmol/ml phospholipids. The tube was vortexed at least 3 times for 15 seconds until all lipids had been suspended. Liposomes were generated by sonicating the lipid suspension for 4 times 15 seconds on ice using an ultrasonic probe (MSE Soniprep 150, London, UK).

In a typical experiment, 50 µl of the liposome suspension was incubated with 20 µg of recombinant GAPR-1 (Serrano et al., 2004) and lysozyme (100 µg) in 50-NT buffer (75 µl total volume). The samples were incubated for 90 minutes at 37 °C or using indicated conditions. The incubation was stopped by cooling the samples on ice. Sucrose [60% (w/v) in 50-NT] was mixed with the sample to a concentration of 36.5% (w/v) sucrose. The samples were overlayed with 500 µl 25% (w/v) sucrose in 50-NT buffer and subsequently with 100 µl 50-NT buffer.

The samples were centrifuged in a TLA-55 rotor (Beckman, Fullerton, U.S) for 90 minutes at 136,000 x g (4 °C). After centrifugation, liposome-bound protein was collected in 300 µl from the top of the gradient. To assure that liposomes have been floated to the top of the gradient, floated lipids were quantified by a phosphate determination according to Rouser with minor modifications (Rouser et al., 1970). Briefly, 37.5 µl of the floated liposome fraction was taken and 150 µl perchloric acid (70-72%) was added, after which samples were heated at 180 °C for at least 1 h until the sample had become clear. After cooling to room temperature, 625 µl water, 125 µl 2.5% (w/v) heptamolybdate and 125 µl 10% (w/v) ascorbic acid were added. Subsequently samples were warmed to 50-60 °C for 20 minutes, after which at 820 nm absorbance was measured. Prior to analysis of the sample by SDS-PAGE and subsequent coomassie staining, proteins were precipitated by chloroform/methanol to concentrate the sample and to remove liposomal lipids. Briefly, to 225 µl of the floated liposome fraction 775 µl chloroform/methanol (1:2) was added. The sample was mixed and centrifuged for 30 min at 13,000 x g. The supernatant containing liposomal lipids was removed and the pellet containing precipitated protein was air-dried.

Triton X-114 phase separation assay

Triton X-114 (TX114) phase separation was performed as described (Bordier, 1981). Briefly, TX114 (Sigma, St. Louis, U.S.) was condensed 3 times with fresh buffer to remove impurities and a 16% (v/v) stock was made. PI-bound GAPR-1 was made by binding GAPR-1 to PI liposomes and subsequent flotation as described above. For each TX114 assay, 100 µl of a top fraction of a liposome binding assay with PI liposomes was used. As a control, non-bound GAPR-1 was diluted in 100 µl 50-NT

buffer. An equal volume of 1% TX114 (v/v) in 50-NT buffer was added to the samples and the mixture was incubated on ice for 40 min. Phase separation was induced by incubation of the samples for 5 min at 37 °C in a water bath and by subsequent centrifugation for 2 min at 13,000 x g in a microtube centrifuge, allowing the separation of the detergent phase from the aqueous phase. Both phases were washed once by adding 100 µl 50-NT buffer to the detergent phase and 100 µl 1% TX114 in 50-NT buffer to the aqueous phase, and centrifugation as above. Both aqueous phases and both detergent phases were pooled. To all samples 50-NT buffer was added to a total volume of 300 µl.

Binding of GAPR-1 to radio-labeled PI

Liposomes were made as described above containing 250 nCi L-3-phosphatidyl [U-^{14}C] Inositol (CFA 641, Amersham Biosciences Piscataway, U.S.) with non-labeled PI, phosphatidylcholine (PC) and cholesterol (1:9:3 mol ratio). 50 µl liposomes, 20 µg GAPR-1 and 100 µg lysozyme in 50-NT buffer with 10 mM β-mercaptoethanol were incubated in a total volume of 75 µl for 100 minutes at 37 °C. Liposomes were pelleted for 1 h at 112,000 x g in a TLA-55 rotor (Beckman, Fullerton, U.S). Proteins in the pellet were resolved on a Novex precast 4-12% gradient gel (Invitrogen, Carlsbad, U.S.) and visualized by staining with coomassie blue R-250 (Serva Electrophoresis, Heidelberg, Germany). To enhance the signal, the gel was soaked in N-AMP (Amersham Biosciences, Piscataway, U.S.) and analyzed by autoradiography using Biomax MR films (Kodak, Rochester, U.S.).

SDS-PAGE and Western blot

Unless indicated otherwise, 14% (w/v) polyacrylamide gels were used. Gels were analyzed by staining with coomassie blue R-250 (Serva Eletrophoresis, Heidelberg, Germany), or by Western blotting. For Western blotting an affinity purified polyclonal antibody to GAPR-1 was used as described (Eberle et al., 2002). Peroxidase labeled goat anti rabbit (Nordic Immunology, Tilburg, The Netherlands) was used as secondary antibody. Supersignal West Pico Chemiluminescent was purchased from Pierce (Rockford, U.S.). Molecular masses were estimated by comparison with SeeBlue® Plus2 Pre-Stained Standard molecular mass marker proteins (Invitrogen, Carlsbad, U.S.).

MALDI-TOF analysis

PI-bound GAPR-1 was made using the liposome binding assay as described above. Briefly, GAPR-1 was incubated with liposomes containing PI, PC and cholesterol (1:4:2.19 mol ratio) for 90 minutes at 37 °C and subsequently liposomes were floated on a sucrose gradient. PI-bound GAPR-1 was collected from the upper fraction of the gradient. GAPR-1 (from a stock solution) and PI-bound GAPR-1 were precipitated using HPLC grade chloroform/methanol (1:2 v/v). The pellet was washed 2 times with HPLC grade chloroform/methanol (1:2 v/v) and resuspended in 10 µl MALDI matrix suspension (5 mg/ml sinapinic acid in 50% acetonitrile/0.1% trifluoroacetic acid). One µl of this suspension was spotted on a MALDI target plate and analyzed using an Applied Biosystems 4700 MALDI Proteomics analyzer (Foster City, U.S.) in a positive linear mode, using an m/z 4,000-40,000 mass range with a focus m/z of 17,000. Data were acquired at a 200 Hz laser repetition rate, a laser intensity of 3500, an acceleration voltage of 20 kV and a digitizer bin size of 2.0 ns. In total, data from 9000 shots were used for the final spectrum.

Results

GAPR-1 binds negatively charged lipids.

In cells, GAPR-1 is stably bound to the Golgi membrane (Eberle et al., 2002). To determine the potential contribution of lipids to the binding of GAPR-1, a liposome binding assay was used. GAPR-1 has a relatively high isoelectric point (9.4) due to the presence of several lysine residues that are concentrated in a positively charged patch exposed on the GAPR-1 surface (Serrano et al., 2004). This suggests a mechanism for GAPR-1 to bind to negatively charged lipids. To determine the lipid binding capability of GAPR-1, we made use of a liposome binding assay. Liposomes were made using phosphatidylcholine (PC) and cholesterol in the presence of various other phospholipids. As the myristoyl-moiety of GAPR-1 increases the affinity of GAPR-1 for lipid membranes and thus interferes with the assay, non-myristoylated GAPR-1 (Serrano et al., 2004) was used for the binding experiments. The GAPR-1 binding assay was performed in the presence of an excess of lysozyme, which is similar to GAPR-1 in terms of size and charge (pI). Lysozyme may therefore allow to discriminate between specific and non-specific interactions of GAPR-1 with negatively charged lipids. After incubation, the amount of liposome-bound GAPR-1 was assessed by flotation of the liposomes on a sucrose gradient. The proteins in the top fraction

containing the floated liposomes were resolved by SDS-PAGE and visualized using coomassie blue (figure 1A). GAPR-1 binding to liposomes composed of only PC and cholesterol is minimal. However, in the presence of negatively charged lipids increased binding of GAPR-1 to liposomes is observed with highest affinity for phosphatidic acid (PA).

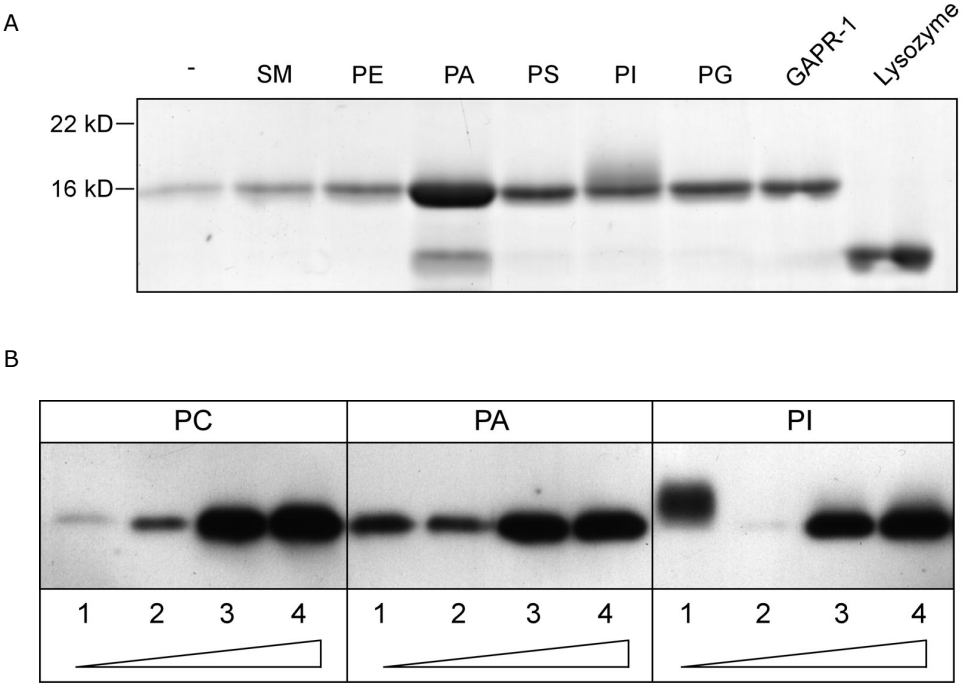


Figure 1. GAPR-1 binds negatively charged lipids.
(A) Affinity of GAPR-1 to liposomes containing different membrane lipids. GAPR-1 and lysozyme were incubated with cholesterol/phospholipid liposomes containing PC as the phospholipid only (-), or 85 (mol) % PC and in addition one of the following lipids (15%): sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI) or phosphatidylglycerol (PG). After incubation at 37 °C for 90 minutes, liposomes were floated on a sucrose gradient. Proteins in the top fraction of the gradient were precipitated with chloroform/methanol (1:2) and subsequently resolved on an SDS-PAGE gel and visualized using coomassie blue. The 16 kD bands represent GAPR-1. Input of GAPR-1 (25%) and lysozyme (25%) are shown on the right (B) GAPR-1 was incubated with liposomes containing PC alone or 90% PC with 10% PA or PI. After the incubation the liposomes were floated on a sucrose gradient. Fractions of the gradient were collected from top (fraction 1, low sucrose) to bottom (fraction 4, high sucrose). Proteins in each fraction were resolved by SDS-PAGE and analyzed by Western blotting with an antibody to GAPR-1.

Lysozyme, however, was not recovered in the liposome fractions. Only in the PA-containing liposomes small amounts of lysozyme can be detected in the liposome fraction, indicating some potential for non-specific interactions under these conditions. After binding to phosphatidylinositol (PI)-containing liposomes, GAPR-1 showed some unexpected behavior. After separation of proteins by SDS-PAGE, GAPR-1 did not migrate as a defined sharp band anymore but as a diffuse protein band caused by reduced migration of some GAPR-1 in the polyacrylamide gel. Western blot analysis confirmed that the diffuse material contained GAPR-1 (see below).

The characteristics of GAPR-1 binding to PA and PI were further investigated. After incubation with PA- or PI-containing liposomes, the liposomes were floated on a sucrose step gradient and each fraction was subsequently analyzed by Western blotting using anti-GAPR-1 antibodies (figure 1B). In agreement with the results shown in figure 1A, GAPR-1 hardly binds to liposomes containing only PC and cholesterol. In the presence of PA or PI, 15-25% (range reflects variations between experiments) of the total amount of GAPR-1 binds to the liposomes. When GAPR-1 is bound to PI-containing liposomes, only GAPR-1 in the top fraction has an increased apparent molecular mass, whereas GAPR-1 in the bottom fractions is not affected by the incubations with PI-containing liposomes. This indicates that only the pool of GAPR-1 that interacts with PI-containing liposomes has changed its physical properties. This is also in marked contrast with the binding of GAPR-1 to PA liposomes, which did not lead to a gel-shift of GAPR-1.

To further characterize the interaction of GAPR-1 with PI-containing liposomes, the temperature dependency of liposome binding of GAPR-1 was investigated. GAPR-1 was incubated with PA- or PI-containing liposomes at various temperatures (4 °C, 20 °C, and 37 °C) (figure 2a). When GAPR-1 is incubated with PA-containing liposomes, the temperature has little effect on the binding characteristics. However, binding of GAPR-1 to PI-containing liposomes is temperature dependent. Upon incubation at 37 °C GAPR-1 binding to PI-containing liposomes is markedly increased. In addition, the molecular weight of GAPR-1 appears higher after incubation at 37 °C as compared to incubations at the two lower temperatures. These data suggest that GAPR-1 binds to PA- and PI-containing liposomes via different mechanisms.

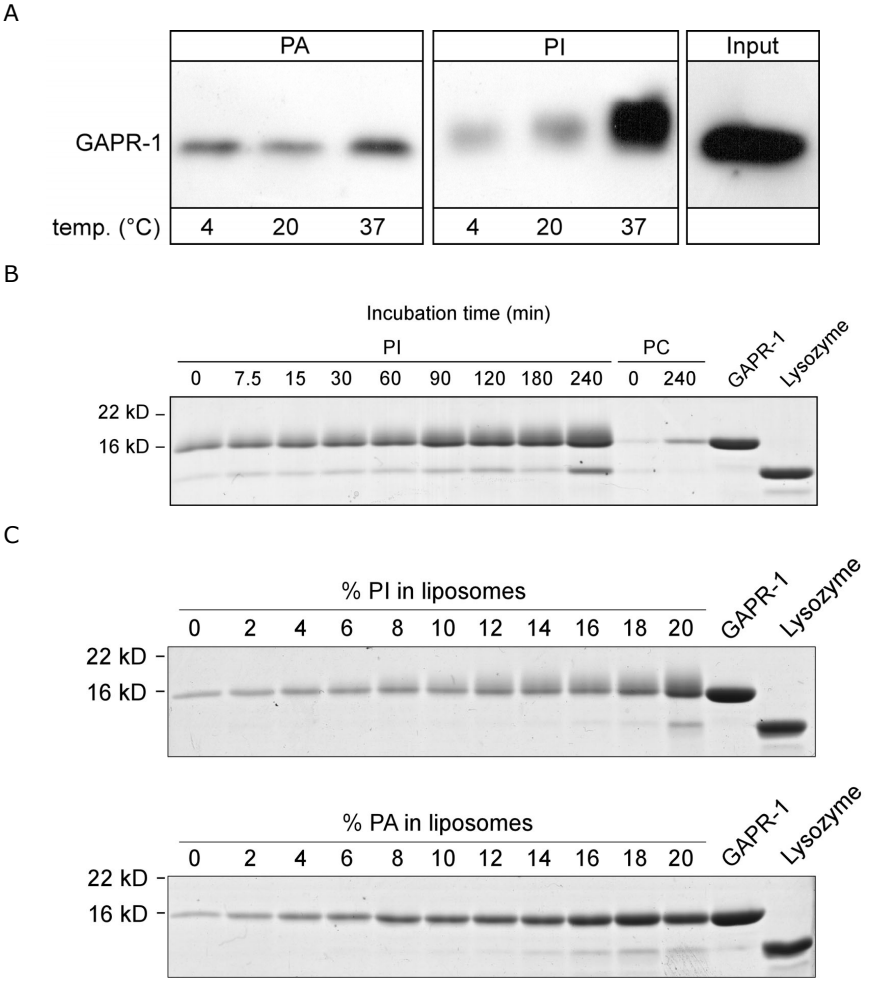


Figure 2. Binding to PI is time, temperature and concentration dependent.
(A) GAPR-1 was incubated with cholesterol/phospholipid liposomes containing 85 (mol)% PC and 15% PA or PI at 4 °C, 20 °C or 37 °C for 90 min, after which the liposomes were floated on a sucrose gradient. The top fractions from the gradient were resolved by SDS–PAGE and analyzed by Western blotting with an antibody to GAPR-1. GAPR-1 from stock is shown in the input lane.
(B) GAPR-1 and lysozyme were incubated with liposomes containing PI during varying time periods as indicated at 37 °C. After flotation of the liposomes on a sucrose gradient, proteins were precipitated with chloroform/methanol. The proteins were visualized using SDS-PAGE and coomassie blue. Input of GAPR-1 (50%) and lysozyme (50%) are shown on the right.
(C) GAPR-1 and lysozyme were incubated with PC/cholesterol liposomes containing varying percentages (in mol phospholipid) of PI or PA as indicated at 37 °C for 90 min. After incubation, the liposomes were floated on a sucrose gradient and bound proteins were visualized using SDS-PAGE and coomassie blue. Input of GAPR-1 (50%) and lysozyme (50%) are shown on the right.

The temperature dependency of the GAPR-1 binding to PI-containing liposomes suggests that this interaction is not solely electrostatic. Additional evidence for this was obtained by determining the time dependency of GAPR-1 binding to liposomes. GAPR-1 was incubated with PC liposomes in the absence or presence of PI for different time periods (0-240 min). After incubation the liposomes were floated on a sucrose gradient and analyzed for the presence of GAPR-1 using coomassie blue staining (figure 2B). In the presence of PI, a time-dependent increase of GAPR-1 binding was observed. Under these conditions, lysozyme did not show a gradual and time-dependent increase. In the absence of PI, GAPR-1 association with liposomes remained minimal. In addition to a gradual increase of GAPR-1 association with PI-containing liposomes, the gel-shift of GAPR-1 can also be observed at early time points, indicating no significant delay of this GAPR-1 modification upon binding to PI-containing liposomes. Lysozyme, however, does not show any gel-shift.

The affinity of GAPR-1 for negatively charged lipids was determined by titrating increasing amounts of PI or PA into PC-containing liposomes in the presence of lysozyme (figure 2C). Both in the presence of PI and PA, GAPR-1 association with liposomes increased with increasing concentrations of negatively charged lipids. The GAPR-1 gel-shift appears independent of the PI concentration since it can be observed at all tested PI concentrations.

GAPR-1 is modified by PI.

A straightforward explanation for the observed gel-shift of GAPR-1 upon binding to PI-containing liposomes is direct binding of GAPR-1 to PI. Therefore, it was investigated whether GAPR-1 becomes modified by PI under experimental conditions that result in a gel-shift of GAPR-1. GAPR-1 is a highly hydrophilic molecule as it has no transmembrane domain and is highly charged at neutral pH (pI 9.4). When modified by a lipid, GAPR-1 is expected to acquire hydrophobic properties, similar to what has been observed for e.g. glycosylphosphatidylinositol(GPI)-anchored proteins. In phase-partitioning experiments, GPI-anchored proteins partition in the hydrophobic (TX114 detergent) phase, but after removal of the GPI-anchor by phospholipase C, they partition in the hydrophilic (aqueous) phase (Bordier, 1981). A similar TX114 partitioning assay was therefore performed using GAPR-1, both before and after binding to PI-containing liposomes. As expected, before binding to PI-containing liposomes the entire GAPR-1 pool partitioned in the aqueous phase (figure 3A). After binding to PI-containing liposomes, however, a large fraction of the GAPR-1 pool partitioned in the detergent phase, indicating a hydrophobic modification of

the protein. The observed gel-shift together with the increase in hydrophobicity suggests stable binding of GAPR-1 to a lipid, most likely PI.

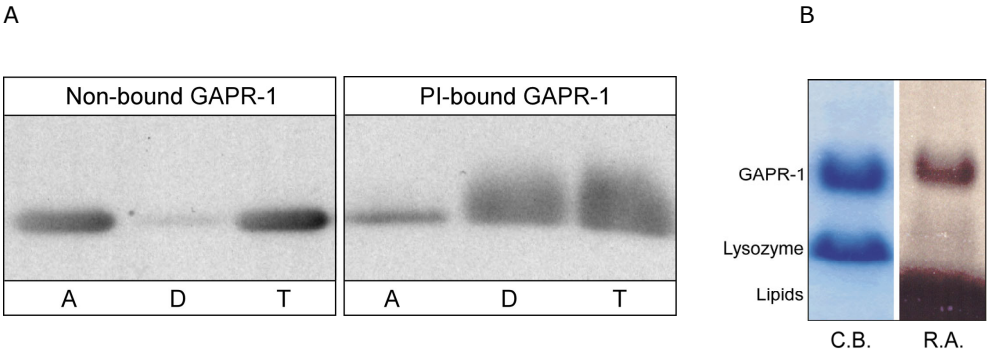


Figure 3. GAPR-1 is modified by PI.
(A) Non-bound GAPR-1 or GAPR-1, which was bound to liposomes containing PI, were extracted with TX114. GAPR-1 in the aqueous phase (A), detergent phase (D) and total (T) were analyzed using Western blot.
(B) GAPR-1 and lysozyme were incubated with liposomes containing radio-labeled PI. After pelleting the liposomes, proteins were resolved on SDS-PAGE and were visualized using coomassie blue (C.B.). Radioactive material in the polyacrylamide gel was visualized by autoradiography (R.A.).

To directly confirm the association of GAPR-1 to PI, GAPR-1, together with lysozyme, was incubated with liposomes containing radiolabeled (^{14}C) PI. After incubation, the liposomes were pelleted and GAPR-1 and lysozyme in the pellet were separated by SDS-PAGE and analyzed by coomassie blue staining and autoradiography. Due to experimental conditions (pelleting instead of flotation of liposomes), more lysozyme was recovered in this fraction. Neither this lysozymal fraction (figure 3B) nor the lysozymal fraction in the supernatant (data not shown) became radioactively labeled. GAPR-1, however, is clearly radioactively labeled, demonstrating that GAPR-1 directly binds to PI, and that this binding is resistant to SDS-PAGE separation and to denaturing conditions (boiling in SDS-PAGE sample buffer prior to SDS-PAGE separation). In addition, before separation by SDS-PAGE, the samples were routinely subjected to chloroform/methanol extraction to remove excess liposomal lipids that interfere with SDS-PAGE protein separation (Materials and Methods). Thus, the binding of GAPR-1 to PI also resists organic extraction procedures, implying strong binding characteristics.

GAPR-1 can bind multiple PI molecules

The TX114 analysis showed an increase in hydrophobicity of GAPR-1 and the autoradiography experiment showed labeling of GAPR-1 with the inositol-headgroup of PI. Together these data suggest binding of the entire PI-molecule to GAPR-1. To show this in more detail using an independent method, GAPR-1 bound to PI-containing liposomes was analyzed by Matrix-assisted LASER desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy. Analysis of GAPR-1 prior to binding to PI-containing liposomes runs at the expected molecular weight of 17.1 kDa.

After binding to PI-containing liposomes, multiple additional peaks were observed with repeating distances of ~0.9 kDa corresponding to the molecular weight of a complete phospholipid with two fatty acids and a headgroup (figure 4). This indicates that a GAPR-1 molecule can bind up to 3 molecules of PI. In contrast to the well defined GAPR-1 peak (figure 4, upper panel), the peaks of GAPR-1 with bound PI-molecules showed peak broadening. This may be explained by the use of PI containing various acyl chains, which add different masses to GAPR-1. It is of note, however, that others have observed broadening of peaks in MALDI-TOF when a lipid with defined acyl chains was coupled to a protein (Gubbens et al., 2007). Probably, the combination of hydrophilic (protein) and hydrophobic (lipid) properties causes interaction with the MALDI matrix resulting in peak diffuseness. The heterogeneous PI binding pattern of GAPR-1 may help explain why PI-bound GAPR-1 runs as a diffuse band during SDS-PAGE rather than a as sharply defined protein band.

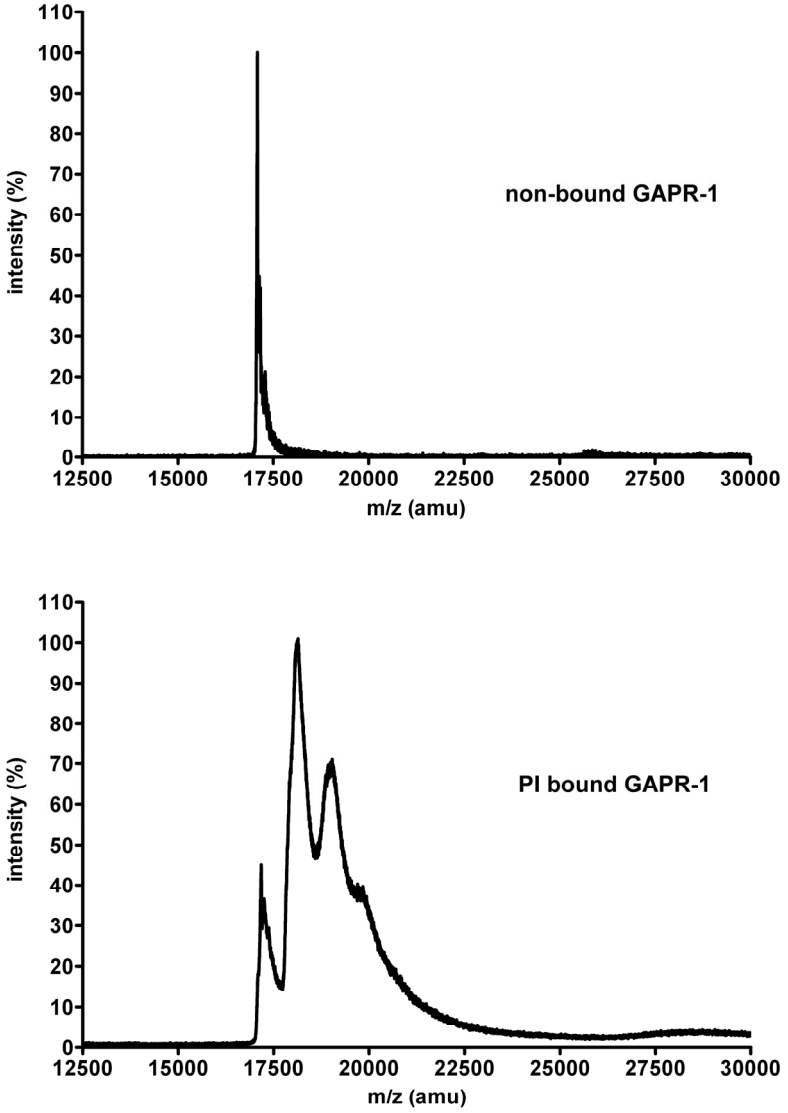


Figure 4. GAPR-1 binds multiple PI molecules.
GAPR-1 was incubated with liposomes containing PI, PC and cholesterol (1:9:3), after which liposomes were floated on a sucrose gradient. Subsequently, proteins were precipitated using chloroform and methanol. Non-bound GAPR-1 was directly precipitated from stock. After this precipitation, the proteins were resuspended in MALDI matrix suspension and loaded onto the mass spectrometer.

Discussion

GAPR-1 interacts with membranes containing negatively charged lipids.

To our knowledge, we have presented the first evidence to show that a member of the superfamily of PR-1 proteins can interact with lipids in biological membranes. All members of this family contain an SCP-domain. Most PR-1 proteins, including the plant PR-1 proteins to which GAPR-1 has most homology, and the CRISP proteins in humans, are secreted. It remains to be established whether the secreted homologues also can interact with lipids. There are indications that the PR-1/SCP family also contains a subset of intracellular proteins. However, their subcellular localization remains to be determined (Kovalick and Griffin, 2005).

Using a liposome binding assay, we show that non-myristoylated GAPR-1 binds to negatively charged lipids. At neutral pH GAPR-1 is positively charged as it has a calculated pI of 9.4 (Eberle et al., 2002). The charge is not equally distributed but is concentrated at one side of the protein molecule, making a positively charged surface (Serrano et al., 2004), which may increase its affinity for negatively charged lipids. This may explain the observed differences in lipid binding between GAPR-1 and lysozyme. Lysozyme does not bind to the liposomes, although it has an even higher pI than GAPR-1 and it is added to the reaction at higher (5x) concentrations than GAPR-1. Whereas PI, PS and PG have a relative charge at pH 7.4 of -1.0, PA has a relative charge of -1.3 (Marsh, 1990) . This charge difference may also explain the observed binding of lysozyme to PA liposomes. Based on these observations, we propose that the affinity of GAPR-1 for lipids may be determined by both charge and structure of the phospholipid head group.

GAPR-1 *in vivo* is strongly bound to membranes (Eberle et al., 2002), whereas in the binding assay *in vitro* only ~10-30% of GAPR-1 binds. This suggests that negative membrane lipids are not sufficient for membrane binding and that GAPR-1 requires additional binding signal(s). GAPR-1 i*n vivo* is myristoylated and this modification, together with the lipid interactions described above, may be sufficient for membrane binding according to the two-signal model of Resh (Resh, 1999). For example, MARCKS proteins which are positively charged proteins with a myristate moiety show stable binding to negatively charged membranes (McLaughlin and Aderem, 1995). Therefore, membrane binding of GAPR-1 *in vivo* may resemble to some extent that of MARCKS proteins.

GAPR-1 binding to PI.

During screening of the affinity for phospholipid classes, it appeared that GAPR-1 binds very strongly to PI. There are proteins known that bind PI. The phosphatidylinositol transfer protein (PI-TP) binds PI facilitating intracellular distribution of PI (Cockcroft and Carvou, 2007; Wirtz, 1991). The cytochrome bc_1 complex is stabilized by PI which is strongly bound via an unusual interhelical bond (Lange et al., 2001). In these cases, the interaction between PI and the protein are mainly based on hydrophobic and electrostatic interactions and the lipid can only be recovered from the protein under native conditions. In the literature there are also few examples of protein-lipid interactions that are partially resistant to strong denaturing conditions. In erythrocytes, Band 3 (AE1) (Maneri and Low, 1989) and glycophorin (van Zoelen et al., 1978) bind multiple phospholipids in a non-covalent but very stable manner, as extraction with chloroform and methanol does not release lipids from glycophorin. Since these proteins contain membrane-spanning domains, interaction with both the lipid head group and the acyl chain is possible.

In contrast to the above described examples, GAPR-1 does not have a transmembrane domain that stabilizes lipid binding via the acyl chain. However, PI binding of GAPR-1 is resistant to chloroform/methanol treatment, TX114 phase separation, SDS-PAGE under reducing conditions and remains intact during mass-spectrometric analysis. This may suggest that GAPR-1 binds to PI via a covalent bond. Covalent linkage of PI to proteins has been described for GPI-anchored proteins. This is a posttranslational modification that requires a C-terminal signal sequence, which is cleaved off and replaced by an GPI-anchor upon membrane translocation [reviewed in (Mayor and Riezman, 2004)]. GAPR-1, however, is not translocated to the lumen of the endoplasmic reticulum and does not contain the signal sequence for GPI-anchorage. To our knowledge, there are only a few examples of cytosolic proteins that are lipidated by a phospholipid. Ubiquitin becomes attached to membranes after baculovirus infection by a novel type of phospholipid anchor (Guarino et al., 1995; Webb et al., 1999). Atg3 couples phosphatidylethanolamine (PE) to Atg8 at the C-terminal glycine via an amide bond (Ichimura et al., 2000). In contrast to PE, PI does not have a amine which makes formation of amide bonds with the protein less likely. In addition, this coupling requires enzymatic activity [Atg3 in the case of PE coupling to Atg8 (Ichimura et al., 2000)], which in the case of the liposome binding assay can only be provided by GAPR-1. Therefore, it remains a formal possibility that GAPR-1 itself may have a catalytic activity. Few examples of such a principle are known. The Hedgehog protein autocatalyzes its binding to

cholesterol (Porter et al., 1996). In this process cysteines are involved, which become irreversibly modified. GAPR-1 contains 2 cysteines while, based on the results from mass spectrometry, 3 PI molecules can bind GAPR-1. Attempts to identify a GAPR-1 peptide with lipid bound to it have so far been unsuccessful (data not shown). Most likely, the hydrophobic modification of peptides results in unexpected behavior of the modified peptide in HPLC analysis and subsequent mass spectrometric analysis in our experimental setup. Although the mechanism is not known, PI-binding of GAPR-1 is time- and temperature-dependent. The temperature dependency may suggest that that membrane fluidity plays a role, as at 37 °C the membrane is more fluid than at 4 °C. The time dependency suggests that the binding of GAPR-1 to PI involves a rate-limiting step. Upon the first binding step (possibly involving weak binding of GAPR-1 to PI via electrostatic interactions), a subsequent step may involve a conformational change of GAPR-1, which is relatively slow.

Possible functions of GAPR-1 lipid modification

Although PE binding of Atg8 mechanistically differs from PI binding of GAPR-1, these modifications may serve the same purpose. When PE is coupled to Atg8, the protein becomes membrane-bound, is clustered and changes its conformation (Ichimura et al., 2004; Ichimura et al., 2000; Kirisako et al., 2000). In an analogous fashion, by binding to PI, GAPR-1 may change its conformation and enter an active or inactive state. In this way PI binding of GAPR-1 may act as a molecular switch. Another possibility is that GAPR-1 is anchored to membranes like GPI-anchored proteins. The anchoring by PI may then facilitate the localization of GAPR-1 to lipid rafts, as GPI-anchored proteins are known to partition into lipid rafts (Simons and Ikonen, 1997).

Judged from Western blotting experiments, GAPR-1 isolated from biological material does not show the gel-shift that was observed in the present study (Eberle et al., 2002). At Golgi membranes, sufficient PI is available to bind GAPR-1. GAPR-1 shows a slight shift already when bound to liposomes containing 4% PI, whereas Golgi membranes contain 6.5% PI (Higgins et al., 1989). This may indicate that GAPR-1 *in vivo* has bound only a fixed number of PI molecules or that PI binding is limited to a small pool of GAPR-1. In addition, it is possible that PI modification is reversible and that cells can uncouple GAPR-1 from PI.

In conclusion, we have shown that GAPR-1 can bind negatively charged lipids and that the binding to phosphatidylinositol bears unusual characteristics. This novel modification may regulate the so far unknown function of GAPR-1.

Acknowledgements

We thank Jos Brouwers for help with initial mass spectroscopic analyses of lipids bound to GAPR-1, Coos Batenburg and Dora Kaloyanova for critically reviewing our manuscript and Ruud Eerland for expert technical help.

Abbreviations

GAPR-1	Golgi Associated plant Pathogenesis Related protein 1
GPI	glycosylphosphatidylinositol
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phophatidylinositol
PR-1	plant pathogenesis related proteins group 1
PS	Phosphatidylserine
SCP	Sperm-coating protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gelelectrophoresis
SM	sphingomyelin
TX114	Triton X-114

References

Baxter, R.M., T.P. Crowell, J.A. George, M.E. Getman, and H. Gardner. 2007. The plant pathogenesis related protein GLIPR-2 is highly expressed in fibrotic kidney and promotes epithelial to mesenchymal transition in vitro. *Matrix Biol*. 26:20-9.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem*. 256:1604-7.

Busso, D., N.M. Goldweic, M. Hayashi, M. Kasahara, and P.S. Cuasnicu. 2007. Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. *Biol Reprod*. 76:701-8.

Cockcroft, S., and N. Carvou. 2007. Biochemical and biological functions of class I phosphatidylinositol transfer proteins. *Biochim Biophys Acta*. 1771:677-91.

Eberle, H.B., R.L. Serrano, J. Fullekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, and J.B. Helms. 2002. Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex. *J.Cell Sci.* 115:827-838.

Eisenberg, I., M. Barash, T. Kahan, and S. Mitrani-Rosenbaum. 2002. Cloning and characterization of a human novel gene C9orf19 encoding a conserved putative protein with an SCP-like extracellular protein domain. *Gene*. 293:141-148.

Gkantiragas, I., B. Brugger, E. Stuven, D. Kaloyanova, X.Y. Li, K. Lohr, F. Lottspeich, F.T. Wieland, and J.B. Helms. 2001. Sphingomyelin-enriched microdomains at the Golgi complex. *Mol.Biol.Cell*. 12:1819-1833.

Guarino, L.A., G. Smith, and W. Dong. 1995. Ubiquitin is attached to membranes of baculovirus particles by a novel type of phospholipid anchor. *Cell*. 80:301-309.

Gubbens, J., P. Vader, J.M. Damen, M.C. O'Flaherty, M. Slijper, B. de Kruijff, and A.I. de Kroon. 2007. Probing the membrane interface-interacting proteome using photoactivatable lipid cross-linkers. *J Proteome Res*. 6:1951-62.

Higgins, J.A., B.W. Hitchin, and M.G. Low. 1989. Phosphatidylinositol-specific phospholipase C of Bacillus thuringiensis as a probe for the distribution of phosphatidylinositol in hepatocyte membranes. *Biochem J*. 259:913-6.

Ichimura, Y., Y. Imamura, K. Emoto, M. Umeda, T. Noda, and Y. Ohsumi. 2004. In vivo and in vitro reconstitution of Atg8 conjugation essential for autophagy. *J Biol Chem*. 279:40584-92.

Ichimura, Y., T. Kirisako, T. Takao, Y. Satomi, Y. Shimonishi, N. Ishihara, N. Mizushima, I. Tanida, E. Kominami, M. Ohsumi, T. Noda, and Y. Ohsumi. 2000. A ubiquitin-like system mediates protein lipidation. *Nature*. 408:488-492.

Kirisako, T., Y. Ichimura, H. Okada, Y. Kabeya, N. Mizushima, T. Yoshimori, M. Ohsumi, T. Takao, T. Noda, and Y. Ohsumi. 2000. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol*. 151:263-76.

Kjeldsen, L., J.B. Cowland, A.H. Johnsen, and N. Borregaard. 1996. SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. *FEBS Lett*. 380:246-50.

Kovalick, G.E., and D.L. Griffin. 2005. Characterization of the SCP/TAPS gene family in Drosophila melanogaster. *Insect Biochem Mol Biol*. 35:825-35.

Kratzschmar, J., B. Haendler, U. Eberspaecher, D. Roosterman, P. Donner, and W.D. Schleuning. 1996. The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. *Eur J Biochem*. 236:827-36.

Lange, C., J.H. Nett, B.L. Trumpower, and C. Hunte. 2001. Specific roles of protein-phospholipid interactions in the yeast cytochrome bc1 complex structure. *EMBO J.* 20:6591-6600.

Li, S., Y. Shin, K.W. Cho, and C.S. Merzdorf. 2006. The Xfeb gene is directly upregulated by Zic1 during early neural development. *Dev Dyn*. 235:2817-27.

Maneri, L.R., and P.S. Low. 1989. Fatty acid composition of lipids which copurify with band 3. *Biochem Biophys Res Commun*. 159:1012-9.

Marsh, D. 1990. CRC Handbook of Lipid Bilayers.

Mayor, S., and H. Riezman. 2004. Sorting GPI-anchored proteins. *Nat Rev Mol Cell Biol*. 5:110-20.

McLaughlin, S., and A. Aderem. 1995. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. *Trends Biochem Sci*. 20:272-6.

Milne, T.J., G. Abbenante, J.D. Tyndall, J. Halliday, and R.J. Lewis. 2003. Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. *J.Biol.Chem.* 278:31105-31110.

Peitzsch, R.M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry*. 32:10436-43.

Porter, J.A., K.E. Young, and P.A. Beachy. 1996. Cholesterol modification of hedgehog signaling proteins in animal development. *Science*. 274:255-9.

Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim.Biophys.Acta*. 1451:1-16.

Rouser, G., S. Fkeischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*. 5:494-6.

Serrano, R.L., A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, and M.R. Groves. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. *J Mol Biol*. 339:173-83.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature*. 387:569-572.

Szyperski, T., C. Fernandez, C. Mumenthaler, and K. Wuthrich. 1998. Structure comparison of human glioma pathogenesis-related protein GliPR and the plant pathogenesis-related protein P14a indicates a functional link between the human immune system and a plant defense system. *Proc.Natl.Acad.Sci.U.S.A*. 95:2262-2266.

Taniguchi, H. 1999. Protein myristoylation in protein-lipid and protein-protein interactions. *Biophys Chem*. 82:129-37.

van Loon, L.C., and E.A. van Strien. 1999. The families of plant pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol*. 55:85-97.

van Zoelen, E.J., A.J. Verkleij, R.F. Zwaal, and L.L. van Deenen. 1978. Incorporation and asymmetric orientation of glycophorin in reconstituted protein-containing vesicles. *Eur J Biochem*. 86:539-46.

Wang, F., H. Li, M.N. Liu, H. Song, H.M. Han, Q.L. Wang, C.C. Yin, Y.C. Zhou, Z. Qi, Y.Y. Shu, Z.J. Lin, and T. Jiang. 2006. Structural and functional analysis of natrin, a venom protein that targets various ion channels. *Biochem Biophys Res Commun*. 351:443-8.

Wang, J., B. Shen, M. Guo, X. Lou, Y. Duan, X.P. Cheng, M. Teng, L. Niu, Q. Liu, Q. Huang, and Q. Hao. 2005. Blocking effect and crystal structure of natrin toxin, a cysteine-rich secretory protein from Naja atra venom that targets the BKCa channel. *Biochemistry*. 44:10145-52.

Webb, J.H., R.J. Mayer, and L.K. Dixon. 1999. A lipid modified ubiquitin is packaged into particles of several enveloped viruses. *FEBS Lett*. 444:136-9.

Wirtz, K.W. 1991. Phospholipid transfer proteins. *Annu Rev Biochem*. 60:73-99.

Yamazaki, Y., H. Koike, Y. Sugiyama, K. Motoyoshi, T. Wada, S. Hishinuma, M. Mita, and T. Morita. 2002. Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. *Eur J Biochem*. 269:2708-15.

Chapter 3

Phytic Acid Induces Alternative Homodimerization of GAPR-1 and Inhibits Membrane Binding to Liposomes

Josse van Galen*, Arie Schouten[$], Ruud Eerland*, Dora Kaloyanova*, Piet Gros[$], and J. Bernd Helms*

*Department of Biochemistry and Cell Biology and Institute of Biomembranes, Utrecht University Utrecht, The Netherlands
[$]Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Faculty of Science, Utrecht University, Utrecht, The Netherlands

Manuscript in preparation

Abstract

Golgi-Associated Plant Pathogenesis-Related protein 1 (GAPR-1) is a mammalian protein that belongs to the superfamily of plant pathogenesis related proteins group 1 (PR-1). GAPR-1 is a peripheral membrane protein that strongly associates with the cytosolic leaflet of Golgi membranes and is enriched in lipid rafts. However, little is known about the mechanism of GAPR-1 interaction with membranes.

Here we report that phytic acid is a potent inhibitor of membrane binding of GAPR-1 to liposomes. Previously, we suggested that dimerization plays a role in the function of GAPR-1 and therefore the effect of phytic acid on the dimerization characteristics was investigated. By size-exclusion chromatography, it was found that phytic acid promotes dimer formation of GAPR-1 in solution. Elucidation of the crystal structure of GAPR-1 in the presence of phytic acid revealed that the GAPR-1 dimer differs from the GAPR-1 dimer formed in the absence of phytic acid. In the presence of phytic acid, the monomeric subunits of the dimer appear to be rotated by 28.3° relative to each other. As a consequence the dimer interface displays a different geometry with different amino acids stabilizing the dimer conformation. Mutation of alanine 68 to a lysine (A68K), an interface contact site unique to the rotated dimer structure, did not prevent the induction of dimer formation by phytic acid. This indicates that mutant GAPR-1 is still capable of binding to phytic acid. However, phytic acid did not inhibit the membrane binding of A68K GAPR-1 to liposomes anymore. The existence of different GAPR-1 dimer conformations may be an important regulatory mechanism of GAPR-1 function.

Introduction

Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) is a mammalian protein, which is a member of the plant pathogenesis related proteins group 1 superfamily. In plants, proteins of this family are characterized by their relatively small molecular mass (14-17 kD), often acidic or basic nature, resistance to proteases and upregulation and secretion upon infection (van Loon and van Strien, 1999). Proteins of this family are related to each other based on sequence homology as well as tertiary structure (van Loon et al., 2006).

The mammalian PR-1 family member GAPR-1 is highly expressed in immune-related tissues and cells (Eberle et al., 2002). Therefore, GAPR-1 may play a role in the innate immune system of mammals. The activity and biological function of GAPR-1 and other PR-1 family members remain unknown. Although an anti-fungal activity has been described (Niderman et al., 1995) other publications report a serine protease activity (Milne et al., 2003). The highly conserved histidine and glutamate pairs of the PR-1 family have also been proposed to represent a catalytic tetrad, although the arrangement showed no similarities to any previously characterized enzymes (Fernandez et al., 1997; Henriksen et al., 2001). We recently suggested that a catalytic triad similar to that of serine proteases may be formed across the dimer interface by residues from both molecules within the dimer (Serrano et al., 2004), implying that dimer dynamics may regulate the activity of the protein.

In contrast to other known PR-1 family members, GAPR-1 is not secreted, but localizes to the cytosolic leaflet of the Golgi membrane (Eberle et al., 2002). GAPR-1 binds very strongly to Golgi membranes, as salt-stripping of membranes or treatment of cells with Brefeldin A, which causes a redistribution of GAPR-1 in cells, do not release GAPR-1 from membranes. GAPR-1 is myristoylated and fatty acid modification could provide a mechanism to anchor this protein to the membrane. The binding energy of myristate incorporation into lipid bilayers is however not sufficient to stably anchor a protein to a membrane (Peitzsch and McLaughlin, 1993). In support of this, several myristoylated proteins do not show exclusive membrane localization and a second interaction is required for efficient membrane binding [reviewed in (Resh, 1999; Taniguchi, 1999)]. GAPR-1 has a pI of 9.4 and therefore GAPR-1 is predicted to have a net positive charge at physiological pH. According to the crystal structure of GAPR-1, several positive charges localize to one protein surface area (Serrano et al., 2004), providing a possibility for efficient electrostatic interactions with negatively charged proteins and/or lipids in the membrane.

Furthermore, GAPR-1 has been shown to interact with caveolin-1, and interactions with other proteins are possible via a coiled-coil domain (Eberle et al., 2002; Serrano et al., 2004). Myristoylation, together with protein-protein interactions and/or electrostatic interactions may be sufficient for the stable membrane binding of GAPR-1.

To gain more insight into the membrane-binding characteristics of GAPR-1, we initially investigated the phospholipid headgroup affinity of GAPR-1 by use of a liposome binding assay (van Galen et al., 2008). During the course of these studies we also searched for biological components that could interfere with the membrane binding of GAPR-1. Here we report that membrane binding of GAPR-1 can possibly be regulated by phytic acid through changes of the dimer configuration of GAPR-1. Therefore, we propose that membrane binding of GAPR-1 may be regulated by a novel regulatory mechanism which involves an intermolecular change in the dimer configuration.

Materials and methods

Reagents

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), L-a-phosphatidylinositol (bovine liver) and cholesterol were purchased from Avanti Polar Lipids. Phytic acid (Cat. no. P8810) was purchased from Sigma (St. Louis, U.S.) and myo-inositol from GIBCO (Grand Island, U.S.). Malonate-imidazol boric acid (MIB), malic acid 2-(N-morpholino)ethanesulfonic acid tris(hydroxymethyl)aminomethane (MMT) were purchased from Molecular Dimensions (Suffolk, U.K.) and poly(ethylene glycol) (PEG) from Fluka/Sigma (St. Louis, U.S.).

Plasmids

pQE60-GAPR-1 WT and pQE60-GAPR-1 Δ4 plasmids were described previously (Groves et al., 2004; Serrano et al., 2004). The GAPR-1 A68K mutant was generated by site-directed mutagenesis using polymerase chain reaction (PCR) on the pQE60-GAPR-1 WT plasmid. The following mutagenic primers were used with the changed codons depicted in bold: 5'-gtgtggggagaacctt**aaa**tgggcatcctatgatc-3' as the sense primer and 5'-gatcataggatgccca**ttt**aaggttctccccacac-3' as the antisense primer. Reactions were performed using *Pfu* polymerase (Fermentas, Burlington, Canada).

The mutated DNA was selected by restriction-site analysis, transformed into *Escherichia coli* (XL-1 blue, Stratagene, Cedar Creek, U.S.), and subsequently amplified using standard molecular biological techniques. The resulting plasmids were verified by sequencing (Baseclear, Leiden, The Netherlands).

Protein purification

For the isolation of both wild type and mutant GAPR-1, a shortened protocol was used as described (Groves et al., 2004). Briefly, *Escherichia coli* (XL-1 blue, Stratagene) were transformed with the pQE60-GAPR-1 WT or pQE60-GAPR-1 A68K plasmid. While shaking at 300 rpm, bacterial cultures were induced with 1 mM IPTG for 4 h, 37 °C. After incubation the bacteria were pelleted, washed twice in in 50-NT buffer (50 mM NaCl, 25 mM Tris pH 7.4), and homogenized by sonication. The homogenate was cleared by centrifugation for 30 min at 14,000 rpm and by subsequent passage through a filter with 20 µm pore size. The soluble protein fraction was passed through a cation exchange column. The column was then washed with 50-NT buffer and GAPR-1 (WT or A68K) was eluted from the column with 350 mM NaCl, 25 mM Tris pH 7.4. The purity of the isolated proteins was confirmed by SDS-PAGE and coomassie blue staining.

Crystal structure determination

GAPR-1 was purified as described (Groves et al., 2004; Serrano et al., 2004). To crystallize GAPR-1 in the presence of phytic acid, 11 mg GAPR-1 (1 mg/ml in 10 mM Tris, 50 mM NaCl, pH 7.4) was incubated for 30 min at 37 °C in the presence of phytic acid (0.7 mM). GAPR-1 was concentrated by use of a spin column (5 kD cut-off filter) (Vivascience, Hannover, Germany) in a cooled centrifuge to a final concentration of 9.6 mg/ml, corresponding to 0.56 mM. Besides GAPR-1 the protein solution contained 50 mM NaCl, 10 mM Tris pH 7.4 and 0.7 mM phytic acid. Initial crystallization screening was performed at room temperature by sitting-drop vapor-diffusion. A HoneyBee 961 (Genomic Solutions) crystallization robot was used for pipetting 50 µl of the reservoir solution into the wells and for combining 150 nl protein solution and 150 nl reservoir solution. Almost immediately after crystallization set-up a shower of microcrystalline needles appeared, that did not grow much larger in time. After several days also a few block-shaped crystals formed in the drops. The initial block-shaped crystals were reproduced and optimized by equilibration of 1+1 µl hanging drops.

Table 1. Data collection and refinement statistics for GAPR-1 with phytic acid

Data collection	
Space group	$C222_1$
Cell dimensions	
a, *b*, *c* (Å)	44.1, 64.9, 103.1
Resolution (Å)	22-1.5 (1.58-1.5)
R_{merge} (%)	8.6 (43.4)
$I / \sigma I$	12.0 (3.4)
Completeness (%)	91.8 (94.7)
Redundancy	4.0 (3.8)
Unique reflections	21757 (3259)
Mosaicity (º)	0.47
Refinement	
Resolution (Å)	36-1.5 (1.54-1.5)
No. reflections	20634 (1551)
No. free-*R* reflections	1116
R_{work} / R_{free}	15.9 (18.4) / 19.1 (26.7)
No. atoms	1459
Residues	1250
Phytic acid	36
Waters	173
Average *B*-factors (Å^2)	
all	10.1
Protein	7.2
Phytic acid	41.6
Water	24.7
R.m.s. deviations	
Bond lengths (Å)	0.012
Bond angles (º)	1.43
Total No. residues	151
Double conformations	Arg37, Ser43, Ser48, Ser58, Ser71, Glu78, Met107, Asn111, Met115, Ser121, Ser127, Glu142
Restrained refinement weighting factor	0.4
TLS group definitions (residue numbers)	4-32, 33-72, 73-103, 104-154

Values in parentheses refer to the highest resolution shell.

Crystals were obtained at various conditions from 100 mM MIB-buffer, MMT-buffer or Tris-buffer pH 6.5-7.0 with 15-25% (w/v) PEG 1500, PEG 3350 or PEG 6000. For data collection, selected crystals were transferred to a cryoprotectant solution and then picked up using a fibre loop prior to flash cooling of the crystal by immersion in liquid nitrogen. In total two data sets were collected: an in-house data set at 1.9 Å resolution (data set 1) and a data set collected at ID23-2 at the ESRF at a resolution of 1.5 Å (data set 2). Data set 1 was collected from a crystal with dimensions 0.08 x 0.04 x 0.04 mm, that was grown using a reservoir solution containing 100 mM MMT-buffer pH 7.0 and 20% (w/v) PEG 6000 and briefly soaked in a cryoprotectant solution with 20% (v/v) glycerol added to the reservoir solution. Data were collected at 100 K on a MAR345 image-plate detector (MAR Research) using Cu $K\alpha$ radiation from a rotating-anode generator (Bruker AXS) operating at 45 KV and 100 mA. Data set 2 was collected from a crystal with dimensions 0.12 x 0.04 x 0.03 mm, that was grown from 100 mM MMT-buffer pH 6.5 and 15% (w/v) PEG 3350. The crystal was soaked for 1 minute in a 2 μl cryoprotectant solution containing 22% (w/v) PEG 1500, 100 mM MMT-buffer pH 6.75 and 20% (v/v) glycerol in the presence of 5 mM phytic acid. Data were collected at 100 K on a CCD detector (MAR Research). The wavelength λ used was 0.8726 Å, the total oscillation was 90º with an 0.5º oscillation step per diffraction frame. Data collection and processing statistics are given in Table 1.

The data were processed with MOSFLM (CCP4, 1994) and scaled using SCALA (CCP4, 1994). The structure from data set 1 was determined by molecular replacement with Phaser (McCoy et al., 2007). The structure of GAPR-1 in the absence of phytic acid (Protein Data Bank entry 1SMB)(Serrano et al., 2004) was used as search model. The refined structure was used as a starting point for the structure from data set 2. The crystal belongs to the orthorhombic space group $C222_1$ with unit cell dimensions a = 44.1, b = 64.9 and c = 103.1 Å. The solvent content calculated was 41% (v/v) with a V_m value of 2.1 Å^3/Da. Manual adjustments of the model were carried out with COOT (Emsley and Cowtan, 2004) and REFMAC5 (CCP4, 1994) was used for subsequent refinements. The electron density for residues 4-152 was clearly interpretable. Residual density at the C-terminus allowed elongation of the polypeptide chain with residues Lys153 and Lys154. However, Lys154 showed poor side-chain density. In addition poor side-chain density was observed for Lys7, Lys30, Lys33, Arg37, Glu78 and Lys88. At the interface of 3 to 4 GAPR-1 molecules the F_o-F_c difference map showed an area of positive electron density (at 3.5-6σ) that was surrounded by several positively charged residues. The

density showed features of phytic acid, but disorder was apparent. We decided to model phytic acid in the density map at half occupancy (the model of phytic acid was from Protein Data Bank entry 1DKP). This model refined well. Surprisingly, despite co-crystallization of GAPR-1 in excess of phytic acid and soaking of crystals in excess phytic acid, the occupation of phytic acid did not improve. Several strong peaks of positive electron density of 3.5-6σ were observed in the difference map near OCS32, Arg37, Gln41, Ser43, Lys113, Lys114, Lys 154 and water 126 that could not be interpreted. In accordance with the 1SMB structure, the cysteinesulfonic acid at residue 32 could be assigned, the density for residue 63 unambigously indicated a cysteine, whereas a cysteinesulfonic acid residue was observed in 1SMB. The final model contained 151 amino acid residues (residues 4-154) corresponding to one monomer of GAPR-1, one phytic acid and 173 water molecules in the asymmetric unit. In total 12 residues have been modeled in two equal populated independent conformations in accordance with the observed electron densities. The final refined model, achieved after subsequent refinements using REFMAC5 with the application of 4 consecutive TLS groups, had an R factor and an R_{free} of 15.9% and 19.1%, respectively. The stereochemistry displayed by the Ramachandran plot shows that 96.2% of the residues fall in the most favored region and 3.8% in the additional allowed region. Statistics on the model quality are given in Table 1. All molecular graphics figures were generated with pymol (W. Delano; http://www/pymol.org/).

Liposome binding assay

Stock solutions of lipids were made in chloroform:methanol (1:2) and stored at -20 °C under N_2 atmosphere. Liposomes were made freshly for each liposome binding assay. To generate liposomes, phosphatidylcholine, phosphatidylinositol and cholesterol in a molar ratio of 1.94:0.34:1 were mixed from stock solutions. The solvents were evaporated using a flow of N_2 with subsequent drying at room temperature in a Speedvac (Savant SVC100H Farmingdale, U.S.) for at least 90 minutes. 50-NT buffer was added to the dried lipid film to a final phospholipid concentration of 17 µmol/ml. The tube was vortexed at least 3 times for 15 seconds until all lipids had been suspended. To create liposomes, the lipid suspension was sonicated 4 times for 15 seconds on ice using an ultrasonic probe (MSE Soniprep 150, London, UK). In a typical experiment, 20 µg recombinant GAPR-1, 100 µg bovine serum albumin (BSA) (Roche, Basel, Switzerland) as carrier protein, 20 µl of the liposome mixture, and the indicated concentrations of phytic acid were incubated in 50-NT buffer (90 µl total volume) for 90 minutes at 37 °C. The incubations were

stopped by cooling the samples on ice. Sucrose [60% (w/v) in 50-NT] was mixed with the samples to a final concentration of 36.5% (w/v) sucrose. The samples were overlayed with 500 µl 25% (w/v) sucrose in 50-NT buffer and subsequently with 100 µl 50-NT buffer and centrifuged in a TLA-55 rotor (Beckman, Fullerton, U.S) for 90 minutes at 55,000 rpm, 4 °C. After centrifugation, protein bound to liposomes was collected in 300 µl from the top of the gradient.

For some experiments (as indicated in the text), liposomes were not floated but diluted in 50-NT (total volume 300 µl) and pelleted at 100,000 x g for 1 h at 4 °C.

Size-exclusion chromatography

Dimerization of GAPR-1 or GAPR-1:phytic acid complexes was determined by gel filtration. Briefly, a 30/10 Pharmacia column containing Superdex 200 (Pharmacia, Uppsala, Sweden) was equilibrated with 50-NT buffer (50 mM NaCl, 25 mM Tris pH 7.4). To estimate the size of the GAPR-1-complexes, myoglobulin (18 kD), ovalbumin (45 kD), BSA (67 kD) and bacitracin (1.4 kD) were used as reference proteins (ICN Biomedicals, Aurora, U.S.). GAPR-1 was incubated in 50-NT buffer with or without 1 mM phytic acid in 100 µl total volume for 30 min at 37 °C. The protein solution was then cooled to 4 °C, after which it was loaded on the column and eluted at 0.5 ml/min at 4 °C. The eluent was collected in fractions of 0.5 ml. The protein content of the fractions was determined by absorbance at 280 nm. Elution of GAPR-1 was determined by Western blotting using a polyclonal GAPR-1 antibody (Eberle et al., 2002). Elution of phytic acid was measured by phosphate determination using a modified spectrophotometric protocol according to Rouser (Rouser et al., 1966). Briefly, 400 µl of each fraction was taken and water was evaporated by heating. The residue was resuspended in 150 µl 70% perchloric acid and heated to 180 °C for 1 h. Samples were cooled and then incubated with 625 µl H_2O, 125 µl 2.5% ammoniumheptamolybdate and 125 µl 10% (w/v) ascorbic acid at 55 °C for 20 minutes. Absorbance of the samples was measured at 820 nm wavelength and compared with that of standard solutions of phytic acid.

Gel electrophoresis

Proteins were resolved on 14% polyacrylamide gels. Gels were analyzed by staining with coomassie blue R250 (Serva Electrophoresis, Heidelberg, Germany) or by Western blotting. In the case of coomassie blue staining, protein amounts were determined by optical scanning of the coomassie blue-stained bands and analysis by use of Quantity One software (Biorad, Hercules, U.S.). The binding of GAPR-1 to

liposomes in the absence of phytic acid was used as a reference (100%). For Western blotting, an affinity-purified polyclonal rabbit antibody against GAPR-1 was used as described (Eberle et al., 2002). Peroxidase-labeled goat anti-rabbit (Nordic Immunology, Tilburg, The Netherlands) was used as secondary antibody. Supersignal West Pico Chemiluminescent was purchased from Pierce (Rockford, U.S.). Chemiluminescence was captured with a Chemidox XRS camera (Biorad) and signals were quantified using Quantity One software package (Biorad). Signals from the incubations were compared to standard curves with known amounts of GAPR-1.

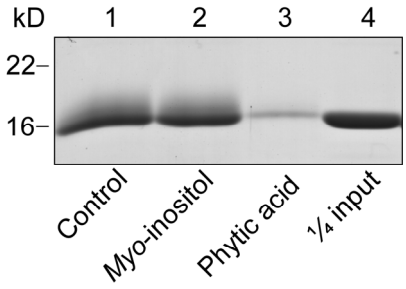
Results

Phytic acid inhibits binding of GAPR-1 to phosphatidylinositol

In cells, GAPR-1 is strongly bound to membranes (Eberle et al., 2002). To investigate the contribution of lipids in GAPR-1 membrane binding, a liposome binding assay was performed which showed that GAPR-1 binds to phosphatidylinositol (PI) but not to phosphatidylcholine (PC) (van Galen et al., 2008). To investigate the contribution of the inositol headgroup, a systematic screen of potential effectors of the GAPR-1 liposome binding assay was performed. After incubation, the liposome-bound GAPR-1 was floated on a sucrose gradient. Liposome-bound proteins in the top fraction were resolved by SDS-PAGE and visualized using coomassie blue. When GAPR-1 was incubated with PI-containing liposomes for 90 min at 37 °C, approximately 20% of GAPR-1 bound to liposomes (Fig. 1A). The observed smearing is due to the strong binding of GAPR-1 to PI that is retained during the denaturing conditions of SDS-PAGE analysis (van Galen et al., 2008). When GAPR-1 was incubated with the liposomes in presence of inositol, the binding of GAPR-1 to the liposomes was not affected. However, when GAPR-1 was incubated with the liposomes in presence of phytic acid (inositol hexakisphosphate, IP6), binding to liposomes was strongly reduced (Fig. 1A).

To determine the affinity of phytic acid for interference with the binding of GAPR-1 to PI-containing liposomes, GAPR-1 was incubated with PI liposomes with increasing concentrations of phytic acid (0 – 3000 µM) (Fig. 1B, top panel). Quantification of the GAPR-1 signals by densitometry showed that the binding of GAPR-1 to PI was inhibited by 50% at 50-60 µM phytic acid (Fig.1B, lower panel). The determined half-maximal inhibition varied in different experiments between 30-70 µM (data not shown).

A



B

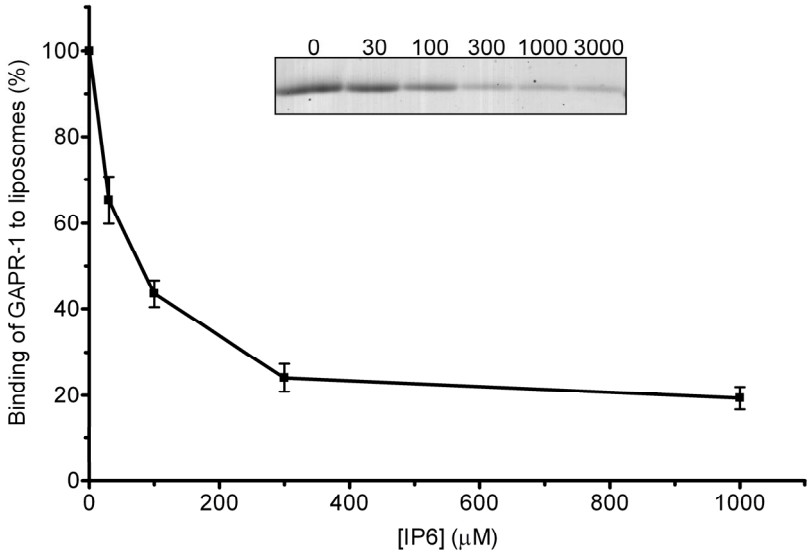


Figure 1. Membrane binding of GAPR-1 is inhibited by phytic acid
(A). Competition assay for GAPR-1 binding to PI-containing liposomes. GAPR-1 was incubated with liposomes (containing PI, PC and cholesterol), bovine serum albumin (BSA) (carrier protein) and buffer in the absence (lane 1) or presence of 5 mM inositol (lane 2) or 5 mM phytic acid (lane 3) (for details, see Materials and Methods). Proteins bound to liposomes (see Materials and Methods) were resolved by SDS-PAGE and stained with coomassie blue. The apparent increase in molecular mass (that results in smearing on the gel) is due to the presence of PI bound to GAPR-1. This lipid binding resists denaturing conditions of gel electrophoresis in the presence of SDS (van Galen et al., 2008).
(B). Titration of phytic acid. Phytic acid was titrated (0-3000 μM) in the GAPR-1 binding assay to PI-containing liposomes under conditions as described for (A). Proteins bound to liposomes (see Materials and Methods) were resolved by SDS-PAGE and stained with coomassie blue. The insert shows a typical experiment over the entire range of titration. To determine half-maximal inhibition, the intensity of the coomassie blue-stained bands were quantified by optical scanning as described in Materials and Methods and plotted as binding percentage relative to the binding in the absence of phytic acid. Error bars represent the standard deviation.

Phytic acid induces dimerization of GAPR-1

Phytic acid is known to promote oligomerization of arrestin 2 and trimerization of the HIV Gag protein (Datta et al., 2007; Milano et al., 2006). Interestingly, dimerization has been implicated in the function of GAPR-1 (Serrano et al., 2004). Therefore, it was investigated whether GAPR-1 also dimerizes or oligomerizes in the presence of phytic acid. To test this, GAPR-1 was incubated with phytic acid or with buffer alone and the size of the GAPR-1 complex was estimated by size-exclusion chromatography. When GAPR-1 was incubated without phytic acid, the protein peak eluted from the column as a protein with an apparent molecular mass of 10 kD, based on comparison with the delay of reference proteins on the column (Fig. 2, top panel). The apparent molecular mass of GAPR-1 is somewhat different from its theoretical molecular mass (17 kD). The delay of GAPR-1 is largely independent of the column resin used, as it was also observed with different types of column material (data not shown). When incubated with phytic acid, GAPR-1 migrates faster on the column, with an apparent molecular mass of 20 kD. This indicates that phytic acid promotes dimerization of GAPR-1. Some GAPR-1 eluted in fractions migrating faster on the column as indicated by the shoulder on the peak. Therefore, GAPR-1 may also form larger complexes in the presence of phytic acid.

To exclude a formal possibility that the shift from 10 kD to 20 kD is caused by non-specific electrostatic binding of a large number (15 molecules of phytic acid correspond to about 10 kD) of phytic acid molecules to GAPR-1, the stoichiometry of the phytic acid-GAPR-1 interaction was determined by measuring the phosphate and protein content in the eluted fractions of the gel-filtration experiments. In the peak fraction a 1:1 ratio of protein:phytic acid was found (data not shown), demonstrating that the shift of phytic acid-bound GAPR-1 is not caused by binding multiple phytic acid molecules to GAPR-1.

A GAPR-1 Δ4 mutant with enhanced dimer characteristics binds strongly to membranes.

The induced dimerization of GAPR-1 by phytic acid and the inhibitory effect on liposome binding suggested that membrane binding of GAPR-1 may be regulated by dimerization. A small fraction (9%) of recombinant and non-myristoylated wild type GAPR-1 (GAPR-1 WT) is present as a dimer in solution (Serrano et al., 2004). In contrast, a GAPR-1 Δ4 mutant, in which 4 amino acids are mutated to alanine, showed an increased dimer formation (78%) in solution (Serrano et al., 2004).

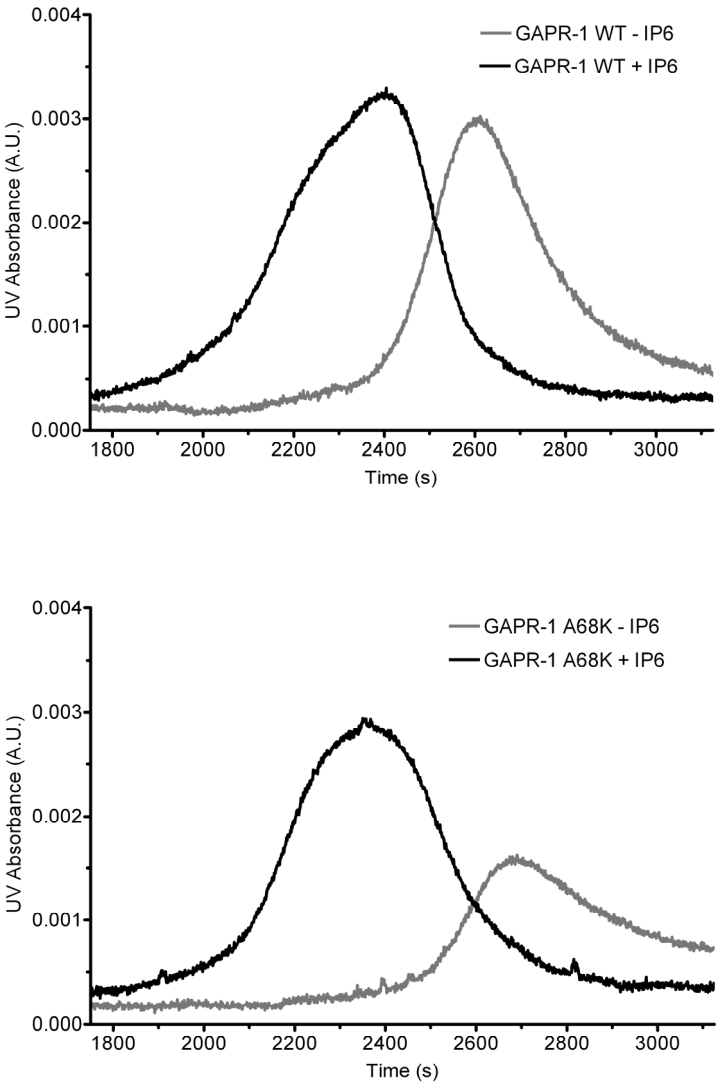


Figure 2. GAPR-1 forms dimers in the presence of phytic acid.
Size-exclusion chromatography of GAPR-1 WT (upper panel) and GAPR-1 A68K (lower panel) in the absence and presence of phytic acid. GAPR-1 (40 µg) was incubated in buffer in the absence (gray line) or presence (black line) of phytic acid (1 mM) for 30 min at 37 °C. The samples were resolved by size-exclusion chromatography as described in Materials and Methods. Proteins eluted from the column were detected by UV absorption. To calibrate the column, ovalbumin, BSA, myoglobin chymotrypsin, and bacitracin were used as reference proteins

If dimerization *per se* is inhibitory for membrane binding, the GAPR-1 Δ4 mutant is expected to bind membranes less well than GAPR-1 WT, and the binding of the mutated protein is expected to be more susceptible to phytic acid. Strikingly, the binding of GAPR-1 Δ4 to liposomes was more efficient as compared to GAPR-1 WT (data not shown). To investigate the effect of phytic acid, the IC_{50} of phytic acid on the binding of the GAPR-1 Δ4 mutant was determined and compared with that of GAPR-1 WT. As shown in Fig. 3, inhibition of the binding of GAPR-1 Δ4 did not reach 50% even at the highest phytic acid concentrations. These data indicate that dimerization itself is not the mechanism of inhibition of GAPR-1 membrane binding.

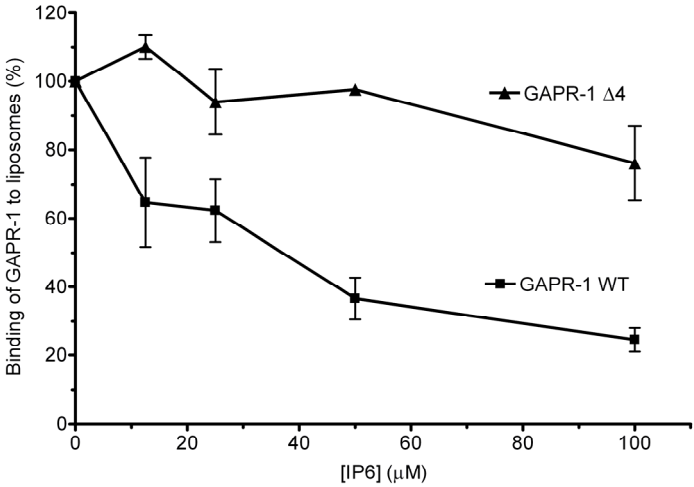
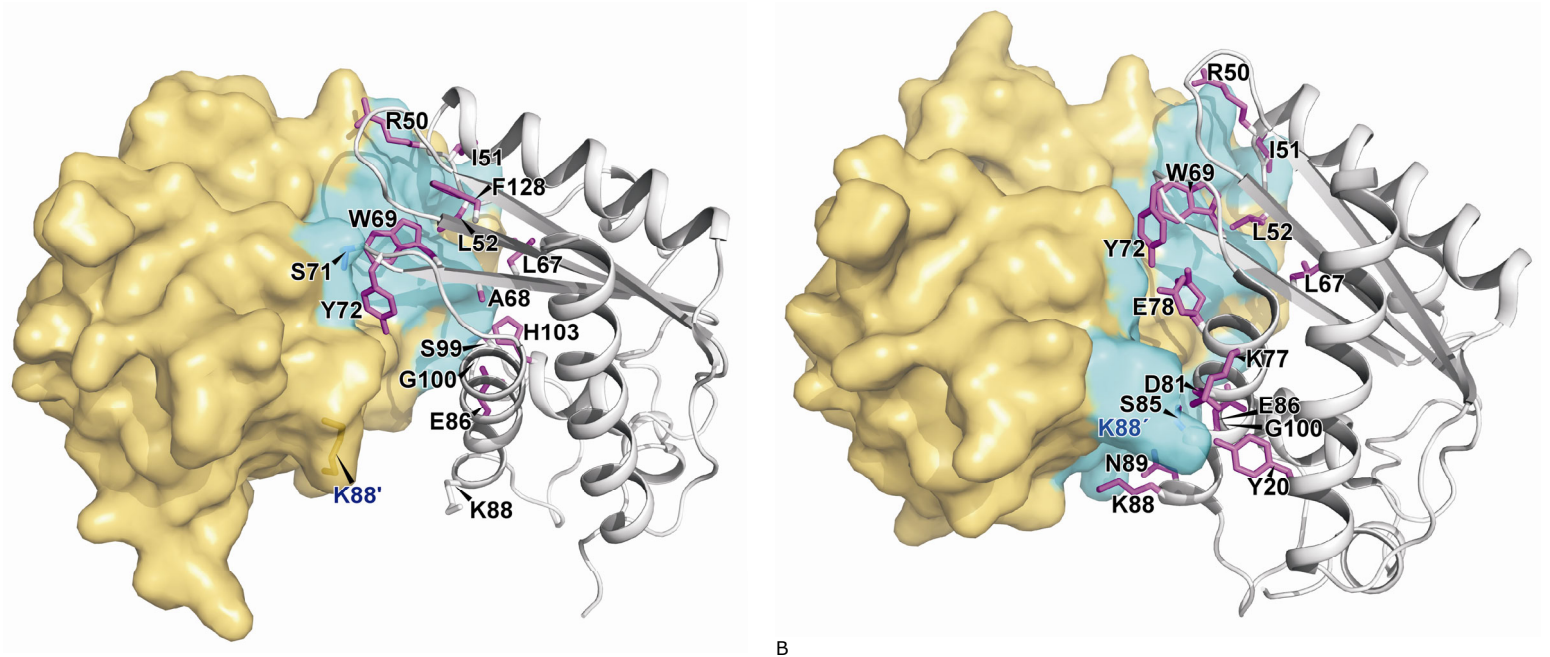


Figure 3. Effect of phytic acid on binding of Δ4-GAPR-1 mutant
GAPR-1 WT (■) or GAPR-1 Δ4 (▲) were incubated with liposomes in presence of phytic acid (0-100 μM) under conditions as described in the legend to Fig. 1A. GAPR-1 bound to liposomes was analyzed by SDS-PAGE and quantified by optical scanning (Materials and Methods). Shown is a representative experiment (n=3) performed in duplicate (bars represent standard error of the mean).

The crystal structure of the GAPR-1 dimer in the presence of phytic acid shows rotation of GAPR-1 monomers.

The size-exclusion chromatography experiments on the induction of dimers by phytic acid showed that GAPR-1 interacts very stably with phytic acid, as GAPR-1 dimers persist during gel filtration chromatography using an elution buffer that does not contain phytic acid (Materials and Methods). We used X-ray crystallography to

determine the effect of phytic acid on the GAPR-1 structure. Comparing the structures of the monomeric subunits in the absence and presence of phytic acid resulted in a r.m.s. fit for 149 aligned Cα positions of 0.47 Å, indicating a good structural agreement between GAPR-1 monomers. Relative to the 1SMB structure (the structure of GAPR-1 in the absence of phytic acid (Serrano et al., 2004) the monomeric subunits of the crystallographic dimer (in the presence of phytic acid) are rotated by 28.3º (Fig. 4). As a consequence the interface has become smaller, resulting into a more open dimeric structure. The surface area buried in the 1SMB dimer [calculated by PISA (Krissinel and Henrick, 2007)] was 943 $Å^2$ (11.7%) and for the structure in presence of phytic acid 712 $Å^2$ (8.6%). Residues observed at the dimer interface in both structures are: Arg50, Ile51, Leu52, Leu67, Trp69, Tyr72, Glu86 and Gly100. In addition, we observe contacts in the open dimer between Ser71 and Ser99' (the dimeric partner molecule is indicated by a ') and His103', between Trp69 and Ala68' and Trp69', between Tyr72 and Ser99' and between Leu52 and Phe128', that was not observed in the closed dimer arrangement. Other residues (contacts between Ser85 and Ser85', between Lys88 and Tyr20', Lys77' and Asp81', and between Asn89 and Glu78') involved in the interface of the closed dimer are rotated away yielding a cleft between the monomers. Due to reorientation of the monomers, the putative catalytic triad, consisting of His54, Glu65 and a symmetry-related Ser71 (Serrano et al., 2004), formed across the dimer interface by residues from both molecules within the dimer, is disrupted. The distance between His54 $N^{\varepsilon 2}$ and Ser71 O^{γ} of the partner monomer is significantly changed from 4.2 Å (in 1SMB) to 5.6 Å; with all intramolecular distances of the putative catalytic residues being the same. The major change in orientation of the side chain of Lys88 is striking. In the closed dimer both symmetry-related side chains of Lys88 seem to close the cleft by sticking out towards their partner monomer, whereas in the structure in the open dimer both symmetry related side chains of Lys88 seem to open the cleft by folding back to their corresponding monomeric subunit (Fig. 4). An additional buried-surface area of 243 $Å^2$ was calculated with PISA for phytic acid. Phytic acid is surrounded by several positively charged residues belonging to different GAPR-1 molecules (Fig. 5). Both phytic acid and the surrounding positively charged residues show disorder. Residues that make a putative salt bridge with phytic acid are Lys7 and Lys33 (molecule 1), Lys53 (molecule 2), Lys88 and Lys154 (molecule 3), and Arg50 (molecule 4). Of the former residues Lys33, Arg50 and Lys88 interact less strong with phytic acid, but could possibly contribute additionally to the ligand binding site.



A B

Figure 4. Representation of the GAPR-1 dimers.

Dimers in the presence (left panel) or absence (right panel) of phytic acid, showing the relative rotation of the subunits of the dimers. Phytic acid is omitted from the figure in the left panel. The monomers of the crystallographic dimers are packed face-to-face. At the interfacial region the molecular surface representation is shown in cyan and the involved residues of the dimeric partner molecule are indicated as sticks in magenta and labeled accordingly. In both panels, the left monomer (yellow) is positioned in the same orientation to allow visualization of the rotation of the partner monomer (right monomer in both panels). Of note, in the presence of phytic acid, residues Lys153 and Lys154 could be assigned, resulting in a small extended structure [left panel, near the bottom of the left (yellow) monomer]. In the left panel both Lys88 residues (not involved in the interface) are pointed out (drawn as sticks and labeled accordingly), showing the major change in orientation of the corresponding side chains, resulting in the entire opening of the cavity.

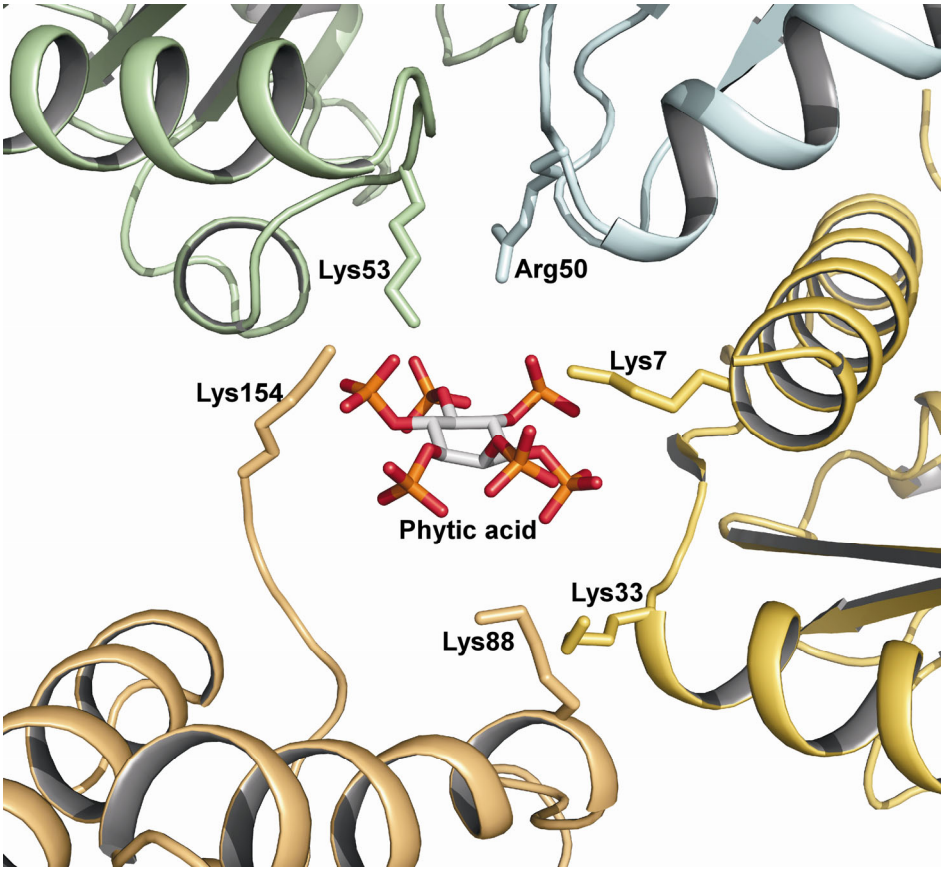


Figure 5. Phytic acid binding site.
Phytic acid modeled in the ligand-binding site. Residues involved (drawn as sticks and labeled accordingly) in salt bridges are Lys7, Lys53 and Lys154; additional weaker bonds are with neighbouring residues Lys33, Arg50 and Lys88. The indicated GAPR-1 molecules are visualized by using different colors.

To investigate whether these lysine residues are involved in the binding of phytic acid to GAPR-1, a GAPR-1 mutant was made in which Lys7, Lys53 and Lys88 were mutated to serine and Lys153 and Lys154 were deleted using site directed mutagenesis (GAPR-1 Q4 mutant). Membrane binding of the GAPR-1 Q4 mutant to PI-containing liposomes was, however, still inhibited by phytic acid (data not shown). In addition, when the Q4 mutant was analyzed by gel filtration, it appeared that the

mutant still dimerized upon binding to phytic acid (data not shown). These results suggest that additional amino acids such as Lys33 and Arg50 are involved in the binding of phytic acid to GAPR-1, or that phytic acid can also bind to other GAPR-1 surface areas that have not been identified in the crystal structure.

Binding of GAPR-1 A68K mutant to PI is not inhibited by phytic acid

An interesting feature of the GAPR-1 crystal structure obtained in the presence of phytic acid is that the monomers are rotated relative to each other in the dimer crystal packing. To investigate the potential significance of this rotation, a GAPR-1 mutant (A68K) was designed and produced which is predicted to be incapable of performing this rotation. Alanine 68 is putatively involved in stabilization of the rotated dimer. When alanine 68 is replaced by lysine, rotation of GAPR-1 monomers relative to each other in the dimer will not be favorable, as the hydrophilic lysine will face a hydrophobic residue of the other monomer. To test whether this GAPR-1 A68K mutant is still able to interact with phytic acid, the behavior of this mutant was analyzed by size-exclusion chromatography (Fig. 2, lower panel). Prior to loading on the column, GAPR-1 WT or GAPR-1 A68K was incubated in the absence or presence of phytic acid. When incubated with phytic acid the GAPR-1 mutant (Fig. 2, lower panel) shows a shift in apparent molecular mass similar as observed for wild type GAPR-1 (Fig. 2 top panel), indicating that the mutant is able to interact with phytic acid and that similar to GAPR-1 WT, phytic acid induces dimer formation of the A68K mutant.

To investigate whether the phytic acid-induced rotation is the cause of the observed inhibition of membrane binding of GAPR-1, a liposome binding experiment was performed with the GAPR-1 A68K mutant in presence or absence of phytic acid. As shown in Fig. 6, the GAPR-1 A68K mutant binds efficiently to liposomes. This is in agreement with its property to form dimers. However, in contrast to GAPR-1 WT the membrane binding of the A68K mutant is not affected by phytic acid. This indicates that membrane binding of GAPR-1 may be regulated by the rotation of monomeric subunits relative to each other in the dimer structure.

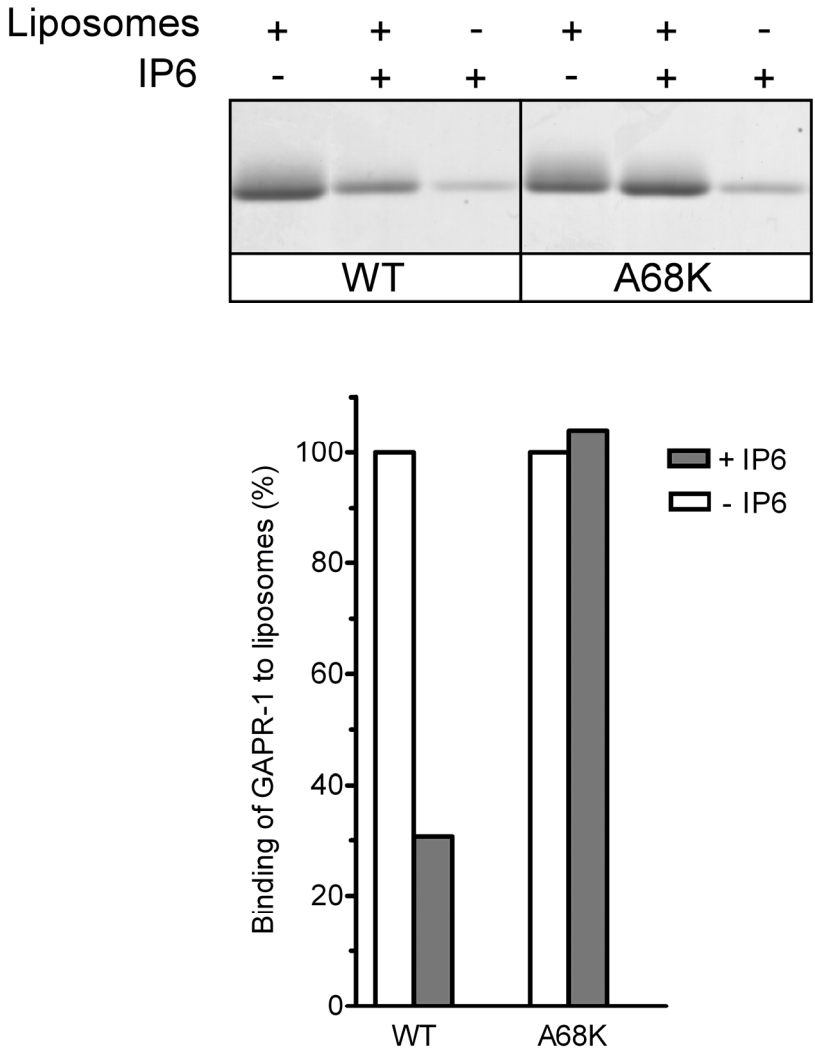


Figure 6. Phytic acid induces dimer formation of GAPR-1 A68K but does not inhibit membrane binding.
Competition assay for GAPR-1 binding to PI liposomes. GAPR-1 WT (lanes 1-3) or GAPR-1 A68K (lanes 4-6) were incubated with liposomes (lanes 1,2,4 and 5) in the absence (lanes 1 and 4) or presence of 1 mM phytic acid (lanes 2,3,5 and 6) under conditions as described in the legend to Fig. 1A. As control, GAPR-1 WT or GAPR-1 A68K was incubated without liposomes (lanes 3 and 6, respectively). GAPR-1 bound to liposomes was analyzed by SDS-PAGE (upper panel) and quantified by optical scanning (lower panel). For details, see Materials and Methods. Shown is a representative experiment (n=3)

Discussion

Phytic acid inhibits membrane binding of GAPR-1 by rotation of monomeric subunits in the GAPR-1 homodimer

In this report we show that membrane binding of GAPR-1 may be regulated by an intermolecular change in the dimer configuration. By gel filtration we show that treatment of GAPR-1 with phytic acid results in the formation of a stable dimer of GAPR-1. Dimerization of GAPR-1 does not inhibit membrane binding per se, as the Δ4 mutant of GAPR-1, which is 80% dimerized in solution, as well as the A68K mutant are fully capable of binding membranes. The 3D model of phytic acid-treated GAPR-1 shows that monomeric subunits of GAPR-1 are rotated relative to each other by 28.3°. To determine whether this rotation is responsible for the observed inhibition of membrane binding, we generated a GAPR-1 A68K mutant in which the rotation of the dimer is destabilized. By replacement of an alanine on the dimer interface for a lysine, it was shown that this mutant was still capable of forming dimers. However, upon addition of phytic acid, membrane binding of the mutant could not be inhibited by phytic acid, indicating that phytic acid inhibits membrane binding of GAPR-1 by rotation of the subunits of the dimer. We favor a model in which GAPR-1 monomers are in a dynamic equilibrium with GAPR-1 dimers. Upon formation of the dimer, the monomeric subunits may be induced to rotate and form an alternative dimeric structure with distinct membrane-binding properties.

GAPR-1 dimerizes in the presence of phytic acid

GAPR-1 has a tendency to form homodimers. A minority (~9%) of recombinant non-myristoylated GAPR-1 in solution is dimerized *in vitro*. Crosslinking experiments showed that GAPR-1 forms homo-dimers on membranes. (Serrano et al., 2004). In addition, GAPR-1 crystallizes as a dimer (Serrano et al., 2004). GAPR-1 *in vivo* also forms homodimers, as GAPR-1 interacts with itself in a yeast 2-hybrid system. We found by size-exclusion chromatography that virtually all GAPR-1 was dimerized in the presence of phytic acid. In the crystal structure, phytic acid does not have a very well defined localization but the data indicated that phytic acid may be located at the interface between three (or even four) GAPR-1 monomers. Although this may suggest that GAPR-1 forms trimers or tetramers, we found by size-exclusion chromatography that the majority of GAPR-1 dimerizes in solution when bound to phytic acid. A small fraction of the GAPR-1 eluting from the gel-filtration column migrated in higher molecular mass fraction, suggesting that a minority of GAPR-1

forms oligomeric complexes. Thus, it is possible that GAPR-1 forms oligomers, or that oligomers are only transiently formed with an equilibrium towards the dimeric complex.

Oligomerization by phytic acid has been observed for other proteins as well. The HIV-1 protein Gag is in a monomer-dimer equilibrium. In the presence of phytic acid this equilibrium shifts towards monomer - trimer (Datta et al., 2007). Phytic acid induces a conformational change in the Gag protein, allowing the termini of the protein to interact with other monomers, thus enabling trimerization (Datta et al., 2007). Trimerization of Gag by phytic acid may be a mechanism *in vivo* to regulate HIV particle size (Campbell et al., 2001). Another example of a protein that is oligomerized by phytic acid is arrestin 2, a regulator of G-coupled receptors (Milano et al., 2006). Oligomerization of arrestin 2 by phytic acid is a regulatory mechanism for its subcellular localization as monomeric arrestin 2 localized to the nucleus and oligomeric arrestin 2 to the cytosol (Milano et al., 2006). Similarly, phytic acid may affect the localization of GAPR-1 by regulating its membrane binding. Together these data imply the presence of an inositol phosphate-mediated switch, regulating the dynamics of proteins (see also below).

Phytic acid and protein function

Phytic acid competes for membrane binding of GAPR-1 in the micromolar range. This is within the range of physiological phytic acid concentrations of mammalian cells (10-100 µM) (Bunce et al., 1993; French et al., 1991; Szwergold et al., 1987). The cytosolic concentration of phytic acid is limited by the concentration of Ca^{2+} and Mg^{2+} ions (≤ 49 µM) and may vary as a function of fluctuations in these ions (Veiga et al., 2006). The concentration of phytic acid in cells is also regulated throughout the cell cycle (Barker et al., 2004). Alternatively, it is possible that phytic acid concentrations show local variations, as suggested by Shears (Shears, 2001). The observed inhibitory effect of phytic acid on GAPR-1 binding to membranes (half maximal inhibition between 30-70 µM) is in close proximity to cytosolic concentrations of phytic acid. Therefore, variations of cytosolic phytic acid concentrations may have a direct effect on GAPR-1 dynamics and function.

In cells, phytic acid is often used as a regulatory molecule or as a cofactor. The stoichiometry of GAPR-1:phytic acid is not known. In the crystal structure the ratio phytic acid:GAPR-1 is 1:1, in close agreement with the stoichiometry after size-exlusion chromatography. For arrestin 2 the phytic acid : protein ratio is concentration dependent. At a low concentration of arrestin 2, it binds 2 phytic acid

molecules occupying both phytic acid binding places. But when arrestin 2 is oligomerized by phytic acid it only binds 1 molecule of phytic acid (Milano et al., 2006). Likewise the stoichiometry of GAPR-1 and phytic acid may vary between different conditions. Irrespective of the actual stoichiometry, phytic acid may function as a cofactor. In yeast, phytic acid localizes to the enzymatic core of the RNA editing enzyme ADAR2 and is required for its activity (Macbeth et al., 2005). In plants, phytic acid was copurified with TIR1, an Fbox protein which functions as a receptor of the plant hormone auxin (Tan et al., 2007). In mammals, phytic acid from the mammalian host cell is important for autocatalytic cleavage of Toxin B from *Clostridium difficile* (Reineke et al., 2007). It is possible that phytic acid is a cofactor for GAPR-1. Dimerization of GAPR-1 results in the formation of a putative catalytic triad (Serrano et al., 2004). Rotation of the dimer by phytic acid may disrupt the catalytic triad, negatively regulating e.g. the putative serine protease activity of GAPR-1.

Phytic acid and inositol phosphate metabolism

Phytic acid plays an important role in regulating vital cellular functions, including cell proliferation and differentiation (Bozsik et al., 2007). Phytic acid has also been proven to be beneficial in preventing and slowing the spread of cancer (Vucenik and Shamsuddin, 2003). Whether the effect of phytic acid on these cellular functions is related to the function of GAPR-1 remains to be established. It also remains to be determined whether GAPR-1 binds phytic acid *in vivo.* There are many examples of proteins that interact with phytic acid *in vitro*, whereas they bind *in vivo* with higher affinity to compounds resembling phytic acid such as inositol phosphates. (Ali et al., 1995; Beck and Keen, 1991; Chaudhary et al., 1998; Frech et al., 1997; Garcia et al., 1995; Hao et al., 1997; Lomasney et al., 1996; Rameh et al., 1997; Schiavo et al., 1996). We have started a systematic screen of all known inositol phosphates and phosphoinositides for their effect on GAPR-1 dimerization/oligomerization and on membrane binding. If such compounds are identified, this would suggest that GAPR-1 dynamics are under the direct control of inositide phosphate signalling cascades. Changes in (specific or local) phosphoinositide concentrations in membranes may affect GAPR-1 dynamics as well.

In conclusion, GAPR-1 binds phytic acid which induces dimer formation and a conformational change in the homodimer. This conformational change leads to a reduced membrane binding of GAPR-1. Thus, dynamics of GAPR-1 dimerization is a novel membrane-binding determinant that may act synergistically with other known

membrane binding determinants such as myristoylation and protein-protein interactions.

Acknowledgements

We thank Chris van de Lest for help with the FPLC and Coos Batenburg for critically reviewing our manuscript. This work was supported by a grant from the Research Council for Earth and life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO).

References

Ali, N., R. Duden, M.E. Bembenek, and S.B. Shears. 1995. The interaction of coatomer with inositol polyphosphates is conserved in Saccharomyces cerevisiae. *Biochem J*. 310 (Pt 1):279-84.

Barker, C.J., J. Wright, P.J. Hughes, C.J. Kirk, and R.H. Michell. 2004. Complex changes in cellular inositol phosphate complement accompany transit through the cell cycle. *Biochem J*. 380:465-73.

Beck, K.A., and J.H. Keen. 1991. Interaction of phosphoinositide cycle intermediates with the plasma membrane-associated clathrin assembly protein AP-2. *J Biol Chem*. 266:4442-7.

Bozsik, A., S. Kokeny, and E. Olah. 2007. Molecular mechanisms for the antitumor activity of inositol hexakisphosphate (IP6). *Cancer Genomics Proteomics*. 4:43-51.

Bunce, C.M., P.J. French, P. Allen, J.C. Mountford, B. Moor, M.F. Greaves, R.H. Michell, and G. Brown. 1993. Comparison of the levels of inositol metabolites in transformed haemopoietic cells and their normal counterparts. *Biochem J*. 289 (Pt 3):667-73.

Campbell, S., R.J. Fisher, E.M. Towler, S. Fox, H.J. Issaq, T. Wolfe, L.R. Phillips, and A. Rein. 2001. Modulation of HIV-like particle assembly in vitro by inositol phosphates. *Proc Natl Acad Sci U S A*. 98:10875-9.

CCP4. 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr*. 50:760-3.

Chaudhary, A., Q.M. Gu, O. Thum, A.A. Profit, Y. Qi, L. Jeyakumar, S. Fleischer, and G.D. Prestwich. 1998. Specific interaction of Golgi coatomer protein alpha-COP with phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*. 273:8344-50.

Datta, S.A., Z. Zhao, P.K. Clark, S. Tarasov, J.N. Alexandratos, S.J. Campbell, M. Kvaratskhelia, J. Lebowitz, and A. Rein. 2007. Interactions between HIV-1 Gag molecules in solution: an inositol phosphate-mediated switch. *J Mol Biol*. 365:799-811.

Eberle, H.B., R.L. Serrano, J. Fullekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, and J.B. Helms. 2002. Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex. *J Cell Sci*. 115:827-838.

Emsley, P., and K. Cowtan. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr*. 60:2126-32.

Fernandez, C., T. Szyperski, T. Bruyere, P. Ramage, E. Mosinger, and K. Wuthrich. 1997. NMR solution structure of the pathogenesis-related protein P14a. *J Mol Biol*. 266:576-93.

Frech, M., M. Andjelkovic, E. Ingley, K.K. Reddy, J.R. Falck, and B.A. Hemmings. 1997. High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J Biol Chem*. 272:8474-81.

French, P.J., C.M. Bunce, L.R. Stephens, J.M. Lord, F.M. McConnell, G. Brown, J.A. Creba, and R.H. Michell. 1991. Changes in the levels of inositol lipids and phosphates during the differentiation of HL60 promyelocytic cells towards neutrophils or monocytes. *Proc Biol Sci*. 245:193-201.

Garcia, P., R. Gupta, S. Shah, A.J. Morris, S.A. Rudge, S. Scarlata, V. Petrova, S. McLaughlin, and M.J. Rebecchi. 1995. The pleckstrin homology domain of phospholipase C-delta 1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry*. 34:16228-34.

Groves, M.R., A. Kuhn, A. Hendricks, S. Radke, R.L. Serrano, J.B. Helms, and I. Sinning. 2004. Crystallization of a Golgi-associated PR-1-related protein (GAPR-1) that localizes to lipid-enriched microdomains. *Acta Crystallogr D Biol Crystallogr*. 60:730-732.

Hao, W., Z. Tan, K. Prasad, K.K. Reddy, J. Chen, G.D. Prestwich, J.R. Falck, S.B. Shears, and E.M. Lafer. 1997. Regulation of AP-3 function by inositides. Identification of phosphatidylinositol 3,4,5-trisphosphate as a potent ligand. *J Biol Chem*. 272:6393-8.

Henriksen, A., T.P. King, O. Mirza, R.I. Monsalve, K. Meno, H. Ipsen, J.N. Larsen, M. Gajhede, and M.D. Spangfort. 2001. Major venom allergen of yellow jackets, Ves v 5: structural characterization of a pathogenesis-related protein superfamily. *Proteins*. 45:438-48.

Krissinel, E., and K. Henrick. 2007. Inference of macromolecular assemblies from crystalline state. *J Mol Biol*. 372:774-97.

Lomasney, J.W., H.F. Cheng, L.P. Wang, Y. Kuan, S. Liu, S.W. Fesik, and K. King. 1996. Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of phospholipase C-delta1 enhances enzyme activity. *J Biol Chem*. 271:25316-26.

Macbeth, M.R., H.L. Schubert, A.P. Vandemark, A.T. Lingam, C.P. Hill, and B.L. Bass. 2005. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science*. 309:1534-9.

McCoy, A.J., R.W. Grosse-Kunstleve, P.D. Adams, M.D. Winn, L.C. Storoni, and R.J. Read. 2007. Phaser crystallographic software. *J Appl Cryst*. 40:658-674.

Milano, S.K., Y.M. Kim, F.P. Stefano, J.L. Benovic, and C. Brenner. 2006. Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding. *J Biol Chem*. 281:9812-23.

Milne, T.J., G. Abbenante, J.D. Tyndall, J. Halliday, and R.J. Lewis. 2003. Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. *J Biol Chem*. 278:31105-31110.

Niderman, T., I. Genetet, T. Bruyere, R. Gees, A. Stintzi, M. Legrand, B. Fritig, and E. Mosinger. 1995. Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against Phytophthora infestans. *Plant Physiol*. 108:17-27.

Peitzsch, R.M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry*. 32:10436-43.

Rameh, L.E., A. Arvidsson, K.L. Carraway, 3rd, A.D. Couvillon, G. Rathbun, A. Crompton, B. VanRenterghem, M.P. Czech, K.S. Ravichandran, S.J. Burakoff, D.S. Wang, C.S. Chen, and L.C. Cantley. 1997. A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J Biol Chem*. 272:22059-66.

Reineke, J., S. Tenzer, M. Rupnik, A. Koschinski, O. Hasselmayer, A. Schrattenholz, H. Schild, and C. von Eichel-Streiber. 2007. Autocatalytic cleavage of Clostridium difficile toxin B. *Nature*. 446:415-9.

Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta*. 1451:1-16.

Rouser, G., A.N. Siakotos, and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids*. 1:85-86.

Schiavo, G., Q.M. Gu, G.D. Prestwich, T.H. Sollner, and J.E. Rothman. 1996. Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. *Proc Natl Acad Sci U S A*. 93:13327-32.

Serrano, R.L., A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, and M.R. Groves. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. *J Mol Biol*. 339:173-83.

Shears, S.B. 2001. Assessing the omnipotence of inositol hexakisphosphate. *Cell Signal*. 13:151-8.

Szwergold, B.S., R.A. Graham, and T.R. Brown. 1987. Observation of inositol pentakis- and hexakis-phosphates in mammalian tissues by 31P NMR. *Biochem Biophys Res Commun*. 149:874-81.

Tan, X., L.I. Calderon-Villalobos, M. Sharon, C. Zheng, C.V. Robinson, M. Estelle, and N. Zheng. 2007. Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature*. 446:640-5.

Taniguchi, H. 1999. Protein myristoylation in protein-lipid and protein-protein interactions. *Biophys Chem*. 82:129-37.

van Galen, J., B. van Balkom, E. Stueven, and J.B. Helms. 2008. Strong binding of GAPR-1 to phosphatidylinositol: Indications for a novel membrane anchor. *Submitted*.

van Loon, L.C., M. Rep, and C.M. Pieterse. 2006. Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol*. 44:135-62.

van Loon, L.C., and E.A. van Strien. 1999. The families of plant pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol*. 55:85-97.

Veiga, N., J. Torres, S. Dominguez, A. Mederos, R.F. Irvine, A. Diaz, and C. Kremer. 2006. The behaviour of myo-inositol hexakisphosphate in the presence of magnesium(II) and calcium(II): protein-free soluble InsP6 is limited to 49 microM under cytosolic/nuclear conditions. *J Inorg Biochem*. 100:1800-10.

Vucenik, I., and A.M. Shamsuddin. 2003. Cancer inhibition by inositol hexaphosphate (IP6) and inositol: from laboratory to clinic. *J Nutr*. 133:3778S-3784S.

Chapter 4

Factors Contributing to Membrane Binding and Subcellular Targeting of GAPR-1: Phosphoinositides, Myristoylation and Phosphorylation

Josse van Galen*, Ramon L. Serrano[$], Dora Kaloyanova*, Ruud Eerland*, J.Bernd Helms*

*Department of Biochemistry and Cell Biology and Institute of Biomembranes, Utrecht University Utrecht, The Netherlands

[$]Department of Biochemistry, Stanford University School of Medicine, Stanford, USA.

Manuscript in preparation

Abstract

Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) is a mammalian protein that belongs to the superfamily of plant pathogenesis related proteins group 1 (PR-1). GAPR-1 strongly associates with the cytosolic leaflet of Golgi membranes and is enriched in lipid rafts. Although the majority of GAPR-1 is localized to the Golgi membranes, some recent evidence suggests that it may also localize to other membranes. The determinants of GAPR-1 membrane binding and cellular localization are unknown. It has been suggested that GAPR-1 membrane binding may be regulated by a combination of factors including a myristoyl anchor, interactions with lipids, and protein-protein interactions.

Here, we report that GAPR-1 binds to negatively charged lipids, most likely due to stereo-specific electrostatic interactions. Although phosphoinositide binding of GAPR-1 shows some degree of promiscuity, the binding efficiency to different phosphoinositides did not correlate with negative charges of the lipid, implying that GAPR-1 recognizes other features of the phosphoinositide molecules. Membrane binding and raft participation of GAPR-1 *in vitro* is enhanced by its myristate moiety. A GAPR-1 mutant defective in myristoylation *in vivo* was partially soluble in the cytosol, but the raft partitioning was not changed, suggesting that myristoylation has a role in membrane anchoring but not in raft participation. Surprisingly, phosphorylation may be involved in membrane localization as a small but distinct pool of highly phosphorylated GAPR-1 could be identified in cytosol. These results suggest that multiple signals act in concert and that changes in each signal may determine membrane binding and subcellular localization of GAPR-1.

Introduction

Golgi-Associated Plant Pathogenesis-Related protein 1 (GAPR-1) is a mammalian protein that is well conserved among vertebrates and is known under various aliases such as GLIPR-2 and c9orf19 (Baxter et al., 2006; Eberle et al., 2002; Eisenberg et al., 2002). GAPR-1 is differentially expressed with high levels of expression in immunocompetent cells and tissues (Eberle et al., 2002). The sequence and tertiary structure of GAPR-1 is highly homologous to that of plant PR-1 proteins, showing that it belongs to the PR-1 superfamily of proteins (Eberle et al., 2002; Serrano et al., 2004). PR-1 proteins are assumed to be involved in plant defense against pathogens, as they are highly upregulated and secreted upon infection of plants by pathogens [reviewed by (van Loon et al., 2006)]. Based on the expression pattern of GAPR-1 and the homology to PR-1 proteins, it was speculated that GAPR-1 may play a role in the innate immune response (Eberle et al., 2002). The biological function and activity of GAPR-1, as well as of the other PR-1 family members, remain unknown.

In contrast to plant PR-1 proteins, GAPR-1 does not contain a signal peptide and therefore it is not secreted via the secretory pathway. Instead, GAPR-1 primarily localizes to sphingomyelin-enriched microdomains or lipid rafts at the cystosolic leaflet of Golgi membranes (Eberle et al., 2002; Gkantiragas et al., 2001). Despite the fact that GAPR-1 is a peripheral membrane protein, it is very strongly bound to membranes. Salt-stripping of isolated membranes or treatment of cells with Brefeldin A, which causes a redistribution of GAPR-1 in cells, does not release GAPR-1 from membranes (Eberle et al., 2002). GAPR-1 is not exclusively localized to the Golgi, as it was also identified in exosomes (Adachi et al., 2006; Pisitkun et al., 2004) (Kaloyanova et al., unpublished). Exosomal localization requires a transient interaction of GAPR-1 with the limiting membrane of late endosomes and subsequent sorting into the internal vesicles (Stoorvogel et al., 2002). In addition, recent evidence indicates that GAPR-1 also associates with phagosomes (Kaloyanova et al., manuscript in preparation). Thus, although GAPR-1 primarily localizes to the Golgi complex under normal conditions, it appears that it may be relocated to other membranes by signals that are currently unknown.

The correct (sub)cellular localization of proteins is essential for their function. Membrane targeting of proteins occurs by membrane-binding signals such as acylation, lipid binding, and by protein-protein interactions. When multiple targeting signals are involved, they may act cooperatively. GAPR-1 *in vivo* is myristoylated (Eberle et al., 2002), which is a stable co- or posttranslational modification (Farazi et

al., 2001) that provides a mechanism for anchoring of GAPR-1 to membranes. The binding energy of myristate incorporation into lipid bilayers is however not sufficient to stably anchor a protein to a membrane (Peitzsch and McLaughlin, 1993; Resh, 1999; Taniguchi, 1999). Therefore, in case of GAPR-1, additional stabilizing factors, such as binding to membrane lipids or membrane proteins are required. GAPR-1 is known to interact with caveolin-1 (Eberle et al., 2002) and it contains a coiled-coil domain, which may facilitate binding to other proteins as well. Furthermore, GAPR-1 is positively charged at physiological pH as it has a calculated pI of 9.4 (Eberle et al., 2002). The charge is concentrated at one area of the GAPR-1 surface (Serrano et al., 2004), which may enable effective binding of negatively charged lipids on the membrane. The relative contribution of the signals described above is, however, unknown.

To gain more insight into the membrane binding characteristics of GAPR-1, we investigated the contribution of myristoylation and phosphorylation of this protein, and its affinity for phosphoinositides to its binding to membranes. We report that GAPR-1 binds to phosphoinositides that have been bound to nitrocellulose membranes or that are incorporated into model membranes. Furthermore, we show that myristoylation of GAPR-1 enhances its membrane binding. Myristoylation *in vitro* affects biophysical properties of GAPR-1, which causes GAPR-1 to be localized to membranes with raft-like properties. However, this effect was not observed *in vivo*, suggesting that protein-protein interactions also play a role in GAPR-1 localization. The presence of phosphorylated GAPR-1 in the cytosol suggests a mechanism to release GAPR-1 from membranes.

Materials and methods

Lipids

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, L-α-phosphatidylinositol (bovine liver), L-α-phosphatidylinositol-4-phosphate (porcine brain-diammonium salt, cat no 840045), L-α-phosphatidylinositol-4,5-bisphosphate (porcine brain-triammonium salt, cat no 840046), 1,2-dioleoyl-*sn*-glycero-3-phosphoinositol-3,4,5-trisphosphate (tetra-ammonium Salt, cat no 850156) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, U.S.). 1,2-Dipalmitoylphosphatidylinositol 3,4-diphosphate (cat no P6990) and L-α-phosphatidyl-D-*myo*-inositol 3-monophosphate,

dipalmitoyl (cat. no P3953) were purchased from Sigma (St. Louis, U.S.). D-*myo*-Phosphatidylinositol 5-phosphate (cat. No P-5016) was purchased from Echelon Biosciences (Salt Lake City, U.S.).

Liposome binding assay

Stock solutions of lipids were made in chloroform : methanol (1:2, v/v) and stored in the freezer at -20 °C under N_2. To generate liposomes, phospholipids and cholesterol were mixed from stock solutions in a molar ratio of 2.28 : 1. The solvent was evaporated using a flow of N_2 with subsequent drying under vacuum overnight at room temperature. 50-NT buffer (50 mM NaCl, 25 mM tris pH 7.4) was added on the dried lipid film to a final concentration of 6.8 µmol/ml phospholipids. The tube was vortexed at least 3 times for 15 seconds until all lipids had been suspended. Liposomes were generated by sonicating the lipid suspension 4 times for 15 seconds on ice using an ultrasonic probe (MSE Soniprep 150, London, UK). In a typical experiment, 20 µg recombinant GAPR-1 (Serrano et al., 2004) and 100 µg bovine serum albumin (BSA) (Roche, Basel, Switzerland) as carrier protein were incubated with 50 µl liposome suspension in 50-NT buffer in a total volume of 90 µl. Samples were incubated for 90 minutes at 37 °C and the incubations were stopped by cooling the samples on ice. Sucrose [60% (w/v) in 50-NT)] was mixed with the sample to a final concentration of 36.5 % (w/v) sucrose. The samples were overlayed with 500 µl 25 % (w/v) sucrose in 50-NT buffer and subsequently with 100 µl 50-NT buffer, after which they were centrifuged in a TLA-55 rotor (Beckman, Fullerton, U.S.) for 90 minutes at 136,000 x g (4 °C). After centrifugation, liposome-bound protein was collected in 300 µl from the top of the gradient.

Liposome recovery by flotation was quantified by a phosphate determination according to Rouser with minor modifications (Rouser et al., 1970). Briefly, 150 µl perchloric acid (70-72%) was added to 37.5 µl of the floated liposome fraction. The samples were heated at 180 °C in a glass tube that was closed with a marble for at least 2 h until the sample had become clear. After cooling to room temperature 625 µl H_2O, 125 µl 2.5% (w/v) ammonium heptamolybdate and 125 µl 10% (w/v) ascorbic acid were added. Then samples were warmed to 50-60 °C for 20 minutes and absorbance was measured at 820 nm.

Prior to analysis of the sample by SDS-PAGE and subsequent coomassie staining, proteins were precipitated by chloroform/methanol to concentrate the sample and to remove liposomal lipids. Briefly, to 225 µl top fraction 775 µl chloroform/methanol (1:2, v/v) was added. The sample was mixed and centrifuged for 30 min at 13,000 x

g. The supernatant containing liposomal lipids was removed and the pellet containing precipitated protein was air-dried.

Protein-lipid overlay assay.

Affinity of GAPR-1 for phosphoinositides was screened by a protein-lipid overlay as described (Johnson et al., 2005). Briefly, nitrocellulose-immobilized phospholipids were obtained from Echelon Biosciences Inc. (Salt Lake City, U.S). The PIP-Strips contained fixed amounts of 100 pmol lipid/spot, the PIP Arrays® contained increasing amounts between 1.6 – 100 pmol lipid/spot, The lipids spotted were lysophosphatidic acid (LPA), lysophosphocholine (LPC), D-*myo*-phosphatidylinositol (PI), D-*myo*-phosphatidylinositol 3-phosphate [PI(3)P], D-*myo*-phosphatidylinositol 4-phosphate [PI(4)P], D-*myo*-phosphatidylinositol 5-phosphate [PI(5)P], L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), D-*myo*-phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2], D-*myo*-phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2], D-*myo*-phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], D-*myo*-phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3], L-α-phosphatidic acid (PA), and L-α-phosphatidylserine (PS).

To prevent non-specific binding, the nitrocellulose-lipid blots were incubated in PBS with 0.05 % (v/v) Tween-20 (PBST) containing 5% (w/v) fatty acid-free BSA [BSA (Roche, Basel, Switzerland), delipidated according to Chen (Chen, 1967)] for 1 h at 20 °C. The blots were then incubated with 0.5 µg/ml purified GAPR-1 (Serrano et al., 2004) in PBST containing 1% (w/v) fatty acid-free bovine serum albumin for 1 h at 20 °C. Lipid blots were washed in PBST (4 times, 5 min each) and incubated for 1 h at 20 °C with an polyclonal rabbit antibody to GAPR-1 (Eberle et al., 2002) in PBST with 1% (w/v) fatty acid free BSA. Blots were washed as before and incubated for 1 h at 20 °C with horseradish peroxidase-labeled goat anti-rabbit IgG (Nordic Immunology, Tilburg, The Netherlands). GAPR-1 bound to the phospholipids immobilized on the membrane were visualized by use of Supersignal West Pico Chemiluminescent (Pierce, Rockford, U.S.) according to the manufacturer's instructions. Chemiluminescence was captured with a Chemidox XRS camera (Biorad, Hercules, U.S.) and signals were quantified using the Biorad Quantity One software package.

Gel electrophoresis

Proteins were resolved on 14% polyacrylamide gels. Gels were analyzed by staining with coomassie blue R250 (Serva, Heidelberg, Germany).

Protein amounts were estimated by coomassie density using Quantity One software of Biorad (Hercules, U.S.). Chemiluminescence was captured with a Chemidox XRS camera (Biorad, Hercules, U.S.) and signals were quantified using Biorad Quantity One software package.

Reconstitution of myristoylated GAPR-1

E.coli were transformed with a plasmid containing N-myristoyl transferase (NMT) [pBB131(NMT)] (Duronio et al., 1990) and pQE60-(GAPR-1 WT) (Groves et al., 2004) and selected for ampicillin and kanamycin resistance (both 50 µg/ml). GAPR-1 was co-expressed with N-myristoyl transferase after induction of 5 ml culture at OD_{600} 0.6 by 1 mM IPTG for 4 h in presence of 75 µCi [9,10]-^{3}H-labeled myristic acid with a specific activity of 54 Ci/mmol (Amersham Pharmacia Biotech). Bacteria were pelleted and resuspended in 50-NT. Bacteria were disrupted by sonication and centrifuged for 30 min at 13,000 x g at 4 °C. Supernatant containing non-myristoylated GAPR-1 was discarded and the pellet containing myristoylated GAPR-1 was dissolved in 100 µl 2% CHAPS in 50-NT and incubated for 1 h at 4 °C. To remove insoluble protein aggregates, the sample was centrifuged at 13,000 x g for 30 min, 4 °C. Supernatant containing solubilized GAPR-1 was mixed with 1.7 µmol phospholipids (10 mol% PI, 90 mol% PC) and 0.75 µmol cholesterol in 100 µl 50-NT with 2% (w/v) CHAPS. To reconstitute GAPR-1 in proteoliposomes, the sample was loaded onto a Sephadex G50 column (Pharmacia, Uppsala, Sweden) that was equilibrated with 50-NT. Proteoliposomes were eluted from the column with 50-NT. Opalescent fractions containing the radioactivity and liposomes were pooled and subsequently used.

Proteoliposomes were incubated for 30 min at 37 °C. Then the samples were cooled on ice and TX-100 was added (1% final conentration). Liposomes were floated on a sucrose gradient as described above (Liposome binding assay) and 300 µl of the top fraction was obtained. 75 µl of the isolated top fraction was used for lipid analysis and determination of [^{3}H] myristic acid incorporation. To this end, the proteins were precipitated by addition of 3 volumes chloroform:methanol (1:2, v/v) and subsequent centrifugation at 13,000 x g for 30 min after which radioactivity was counted in the protein pellet. From the supernatant lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959) and phosphate was measured according to Rouser (Rouser et al., 1970) as described above. Of the remaining top fraction (225 µl), proteins were precipitated with 3 volumes chloroform:methanol (1:2, v/v) as described above. The protein pellet was analyzed by SDS-PAGE. To enhance the

signal, the gel was soaked in N-AMP 100 (Amersham Biosciences, Piscataway, U.S.) and analyzed by autoradiography using Biomax MR films (Kodak, Rochester, U.S.).

Generation of constructs

To facilitate cloning of green fluorescent protein (GFP) into pQE60-GAPR-1 (Groves et al., 2004; Serrano et al., 2004), the existing BamHI restriction site in the construct was eliminated without changing the GAPR-1 amino acid sequence by site-directed mutagenesis. Briefly, reactions were performed using *Pfu* polymerase (Fermentas, Burlington, Canada) and 5'-ctggccagcacgagaatcctcaagcacagc-3' and 5'-gctgtgcttgaggattctcgtgctggccag-3' as sense and antisense primer, respectively. Then template DNA was digested by *DpnI* (Fermentas). The remaining mutated DNA was transformed into *Escherichia coli* (XL-1 blue, Stratagene, Cedar Creek, U.S.) and subsequently amplified using standard molecular biological techniques, and the resulting plasmids verified by sequencing (Baseclear, Leiden, The Netherlands). A new BamHI restriction site was created in the selected region by site-directed mutagenesis as described above using 5'-ggcgtggggaagggatccgcaagtgacgg-3' and 5'-ccgtcacttgcggatcccttccccacgcc-3' as mutagenic primers. GFP cDNA, flanked by BamHI restriction sites, was amplified by PCR from the pEGFP-C1 (Clontech, Palo Alto, U.S.) plasmid carrying a A206K mutation (a kind gift of Dr. J. Lippincott-Schwartz) using 5'-cgcggatccatggtgagcaagggc-3' and 5'-cgcggatcccttgtacagctcgtc-3'. Amplified GFP DNA was restricted with BamHI and cloned into pQE60-GAPR-1 using the new BamHI restriction site as described above. DNA coding for the GAPR-1-GFP chimera was amplified using the following primers: 5'-cggggtaccatgggcaagtcagct-3' and 5'-gccctcgagttacttcttcggcgg-3' and cloned into pCDNA3 using restriction with KpnI and XhoI. The resulting construct was verified by sequencing (Baseclear, Leiden, The Netherlands) and by expression of fluorescent GAPR-1 in HeLa cells. For the generation of non-myristoylated GAPR-1-GFP (Δmyr-GAPR-1-GFP), site-directed mutagenesis was used with the following primers 5'-gggtaccatggccaagtcagcttcc-3' as the sense primer and 5'-gaagctgacttggccatggtacccg-3' as the antisense primer

Cell culture and FACS

HeLa cells were cultured in DMEM (GIBCO/Invitrogen , Paisley, UK) supplemented with 10% fetal calf serum (FCS) (GIBCO/Invitrogen) and penicillin-streptomycin (GIBCO/Invitrogen).

For expression of GAPR-1-GFP, cells were transfected with GAPR-1-GFP WT or Δmyr in pCDNA3 by use of Lipofectamine™ 2000 Reagent (Invitrogen) according to

the manufacturers manual. After overnight expression, approximately $10x10^6$ cells were harvested using trypsin/10x EDTA (Invitrogen) and collected in PBS with 10% FCS. GFP positive cells were selected by fluorescence-activated cell sorting (FACS) (Cytopeia Influx, Seattle, U.S.) using a 100 µm nozzle and 3-6 x 10^6 cells were collected. Cells were pelleted by centrifugation at 340 x g for 5 min followed by resuspension in homogenization buffer (HB, 250 mM sucrose with 10 mM tris pH 7.4). Subsequently the cells were pelleted as described above and were resuspended in HB with protease inhibitors (4 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin A and 0.25 mM PMSF from Sigma). Cells were homogenized by passing them with a 1-ml syringe 20 times through a 26G needle. A post nuclear supernatant (PNS) was obtained by centrifugation of the homogenate at 1,000 x g for 5 min. Protein content of the PNS was determined by use of Coomassie Plus™ Protein Assay (Pierce, Rockford, U.S) according to the manufacturers manual; PNS containing 200 µg protein was separated into a cytosol (supernatant) and a membrane (pellet) fraction by centrifugation at 100,000 x g for 45 min (4 °C). Cytosolic and membrane protein fractions were precipitated using $CHCl_3$/methanol as described above, and analyzed by Western blotting (PVDF membrane Whatmann, Sanford ME, UK). Membranes were probed for GAPR-1 and GFP, secondary antibodies from Nordic (Nordic Immunological Laboratories, Tilburg, NL)

Fluorescence microscopy

Cells were transiently transfected with the GAPR-1-GFP constructs, and were prepared for fluorescence microscopy according to standard procedures. Briefly, cells were fixed in phosphate-buffered saline (PBS) containing 3.5% paraformaldehyde for 30 min, quenched with 50 mM NH_4Cl in PBS, and mounted with Fluoromont-G™ (SouthernBiotech, Birmingham,U.S.). Images were taken with the use of a Zeiss inverted fluorescence microscope equipped with a cooled charge-coupled device camera. The images edited with Canvas 8.0 (Deneba),

Detection of phosphorylated GAPR-1 in vivo

CHO cells in 10-cm plates at 80-90% confluency were washed with 50 mM HEPES buffer pH 7.5 and incubated in 4 ml of phosphate-free DMEM (Sigma) with 20 mM HEPES and 0.25 mCi/ml carrier free $H_3{}^{32}PO_4$ (ICN, Irvine, U.S.) for 4 h at 37 °C. The medium was removed from the plates, the cells were washed twice with cold PBS, harvested and centrifuged at 500 x g for 10 min after which the pellet was resuspended in 25 mM PIPES, pH 6.5, 2 mM EDTA, 150 mM NaCl (PEN buffer). Cells

were homogenized by passing them through a 27G needle. The homogenate was centrifuged at 500 x g for 10 min, and the PNS was collected. The PNS was centrifuged at 100,000 x g for 1 h to separate the sample into a total membrane fraction (pellet) and cytosolic fraction (supernatant). The total membrane fraction was dissolved in PEN buffer with 1% Triton X-100 and incubated for 30 min at 4 °C, followed by centrifugation at 100,000 x g for 1 h. The total membrane fraction was resuspended in 0.1 ml of 1% SDS, Tris-HCl 50 mM, pH 6.8, and heated to 95 °C for 5 min. SDS was quenched by addition of 9 volumes of immunoprecipitation (IP) buffer (1% Triton X-100 in PEN buffer). Immunoprecipitation was carried out using an antibody to GAPR-1 (Serrano et al., 2004), coupled to Sepharose 4B beads (see below). The beads were incubated with both fractions overnight at 4 °C. After incubation, the beads were washed twice with IP buffer, and 4 times with PEN buffer, respectively. The beads were resuspended in 30 µl 56 mM Tris-HCl pH 6.8, 3% SDS, 2.5% β-mercaptoethanol, 5% glycerol, 0.01% bromophenol blue, incubated for 2 min at 95 °C, and centrifuged at 13,000 x g for 30 seconds. The supernatant was analyzed by SDS-PAGE and Western blotting. The membrane was probed with anti-GAPR-1 (Eberle et al., 2002) and HRP-conjugated protein G served to detect bound anti-GAPR-1. After analysis, the membrane was washed several times in PBST buffer dried and phosphorylated GAPR-1 was detected by autoradiography.

Preparation of antibody-coupled beads

10 µl of polyclonal serum against the C-terminus of GAPR-1 (α-1852) was incubated with 50 µl of protein A Sepharose (Fast Flow, Amersham Pharmacia Biotech, Freiburg, Germany) and 50 µl of PBS containing 0.5% milk for 90 min at room temperature. The beads were washed twice with PBS and twice with IP buffer before use.

Results

GAPR-1 binds to phosphoinositides

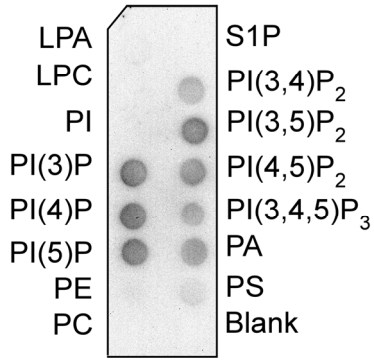
GAPR-1 is predicted to be highly positively charged at physiological pH as it has a calculated pI of 9.4 (Eberle et al., 2002). Most of the positively charged residues of GAPR-1 are located to one surface area of the three dimensional structure of GAPR-1. This positively charged surface patch may be utilized to bind negatively charged lipids. Previously, we showed that GAPR-1 binds to the negatively charged lipids phosphatidylinositol (PI) and phosphatidic acid (PA) (van Galen et al., 2008). The binding to PI is unusual, as it bears several hallmarks of a covalent interaction (van Galen et al., 2008). The binding to PA resembled an electrostatic interaction. Therefore, GAPR-1 may bind to other lipids as well and possibly even stronger when lipids with multiple negative charges, such as phosphoinositides, are involved. To investigate whether GAPR-1 binds phosphoinositides, a protein-lipid overlay assay was performed which allows the screening of a wide array of lipids including all physiological relevant phosphoinositides. GAPR-1 was incubated with an PIP Strip®, which is a nitrocellulose membrane on which 100 pmol of all cellular phosphoinositide classes had been spotted. As the myristoylated GAPR-1 is not soluble, non-myristoylated GAPR-1 was used in this assay

Fig. 1A shows that GAPR-1 binds to all phosphatidylinositol monophosphates, phosphatidylinositol bisphosphates and the phosphatidylinositol trisphosphate tested, albeit with some differences in affinity. In agreement with previous liposome studies (van Galen et al., 2008), GAPR-1 hardly binds the neutral phospholipids phosphatidylcholine (PC), phosphatidylethanolamine and lyso-phosphatidylcholine. GAPR-1 does not bind to all phosphorylated lipids, as binding to sphingosine 1-phosphate was not observed. Remarkably, GAPR-1 binds strongly to PA acid but not to lysophosphatidic acid, although these lipids have the same headgroup. These observations suggest that binding of GAPR-1 to negatively charged lipids is not solely based on electrostatic interactions as i) increasing the negative charge within the same group of lipids does not *per se* result in increased affinity for lipids; and ii) changes in the lipid moiety without changing the net charge results in drastic differences in affinity.

The data generated with PIP Strips represent only relative binding of GAPR-1 binding at one particular concentration to lipids spotted in one particular amount. To investigate the affinity of GAPR-1 for phosphoinositides in more detail, a PIP Array was used. On a PIP Array lipids are spotted in quantities ranging from 1.6 to 100

pmol. Fig. 1B shows that GAPR-1 has affinity for all phosphoinositides, with highest affinity for PI(4)P, PI(5)P, and PI(3,5)P_2.

A



B

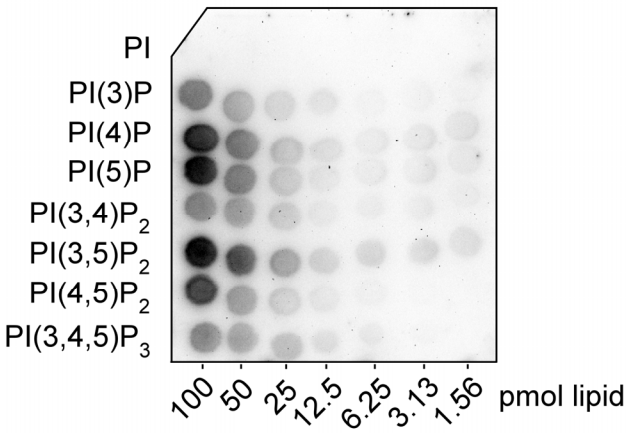


Figure 1: GAPR-1 binds phosphoinositides and PA on nitrocellulose membranes
(A) Phosphoinositide affinity of GAPR-1 was investigated in a protein-lipid overlay assay. Recombinant non-myristoylated GAPR-1 was incubated with nitrocellulose membranes on which lipids were spotted (100 pmol/spot) as described in Materials and Methods. Bound GAPR-1 was detected with a polyclonal antibody to GAPR-1. Chemiluminescence was recorded and quantified. LPA : lysophosphatidic acid, LPC : lysophosphocholine, PI : phosphatidylinositol, PI(3)P : phosphatidylinositol-3-monophosphate, PI(4)P : phosphatidylinositol-4-monophosphate, PI(5)P : phosphatidylinositol-5-monophosphate, PI(3,4)P_2 : phosphatidylinositol-3,4-bisphosphate, PI(3,5)P_2 : phosphatidylinositol-3,5-bisphosphate, PI(4,5)P_2 : phosphatidylinositol-4,5-bisphosphate, PI(3,4,5)P_3 : phosphatidylinositol-3,4,5-triphosphate, PS : phosphatidylserine, PC : phosphatidylcholine, PE : phosphatidylethanolamine, S1P : sphingosine 1-phosphate.
(B) Titration of lipids on a nitrocellulose membrane. Abbreviations as in (A), but lipids were spotted in the range of 1.6 – 100 pmol.

GAPR-1 binding to phosphoinositides in a liposome binding assay.

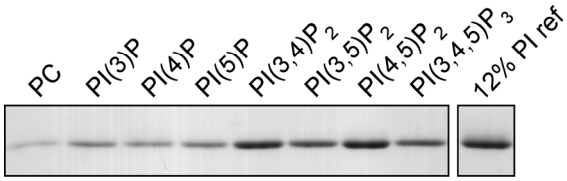
On PIP Strips the lipids bound to a nitrocellulose membrane, but the orientation and/or ordering of the lipids on the membrane is not known. Therefore, the results obtained with the PIP Array were compared to a liposome binding assay, with the lipids present in a bilayer configuration. An additional advantage of liposomes is that they can be incubated under conditions better resembling physiological conditions, i.e. in the absence of detergent and at 37 °C.

To investigate the phosphoinositide affinity of GAPR-1 in the liposome assay, non-myristoylated GAPR-1 was incubated with PC-cholesterol liposomes containing 1 mol % phosphoinositide. GAPR-1 has a very low affinity for PC [Fig. 1 and (van Galen et al., 2008)]. As positive control, PC-cholesterol liposomes containing 12% PI were used (van Galen et al., 2008). After incubation the liposomes were floated on a sucrose gradient and liposome-bound GAPR-1 in the top fraction was analyzed by SDS-PAGE, visualized using coomassie blue (Fig. 2A) and quantified by densitometry (Fig. 2B).

GAPR-1 appears to bind all phosphoinositides tested, as the binding to all phosphoinositide-containing liposomes was stronger than to the liposomes containing PC-cholesterol only. Moreover, GAPR-1 has a higher affinity for phosphoinositides than for PI. For instance, GAPR-1 shows as similar affinity for liposomes containing 1% PI(3,4)P_2, 1% PI(4,5)P_2 and 12% PI (Fig. 2A). The affinity of GAPR-1 differs between phosphoinositides. In the liposome-binding experiment, GAPR-1 has a higher apparent affinity for phosphatidylinositol bisphosphates than for monophosphates. This is in contrast to the results observed for the PIP Strip. There is a possibility that differences in the obtained results between the PIP Strip and liposomes are caused by ordering and clustering of lipids by cholesterol. Therefore, we investigated the detergent-resistance of membranes, which may be an indicator for lipid ordering (Brown and London, 1997). When liposomes were dissolved by 1% Triton X-100 at 4 °C, about one third of the membranes appeared to be detergent-resistant in all liposomes tested (data not shown), which may suggest that the membranes are partially ordered. Cholesterol may promote lipid ordering in model membranes (Brown and London, 1998). Therefore, to exclude the possibility that differences between the liposome-binding experiment and the protein-lipid overlay are caused by lipid clustering, the liposome-binding experiments were repeated with liposomes lacking cholesterol. In contrast to liposomes with cholesterol, clustering of lipids was not detected in the absence of cholesterol, as determined by detergent resistance (data not shown). However, in the absence of cholesterol, GAPR-1 showed

a similar binding pattern to the liposomes with different phosphoinositide compositions, as shown in Fig. 2 (data not shown).

A



B

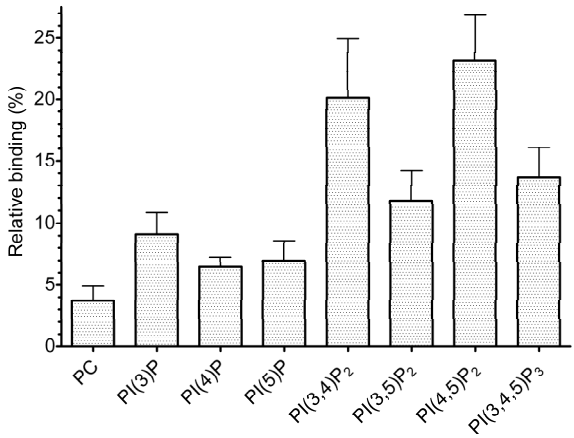


Figure 2: Binding of non-myristoylated GAPR-1 to phosphoinositides in liposomes.
(A) GAPR-1 was incubated with PC-cholesterol liposomes containing 1% phosphoinositide in the presence of BSA as carrier protein. As reference, GAPR-1 was incubated with PC-cholesterol liposomes containing 12% phosphatidylinositol (PI) in the presence of BSA. Liposomes were floated on a sucrose gradient and proteins in the top fraction were resolved by SDS-PAGE and stained with coomassie blue. (B) Quantification of the intensity of GAPR-1 coomassie bands by scanning densitometry. The combined signals of all investigated lipids was set at 100%. In the graph the signal of individual lipids is compared to the total signal (%). The mean of 5 separate experiments is shown, except for PI(4)P, for which the mean of 2 experiments is given. Error bars represent the standard deviation.

Myristoylation contributes to membrane binding of GAPR-1.

Non-myristoylated GAPR-1 binds to membranes *in vitro*, but it is not known whether lipid binding is sufficient for localizing GAPR-1 to membranes *in vivo*. According to the two-signal model as proposed by M. Resh (Resh, 1999), basic proteins that are myristoylated may stably bind to membranes by a combination of protein-lipid

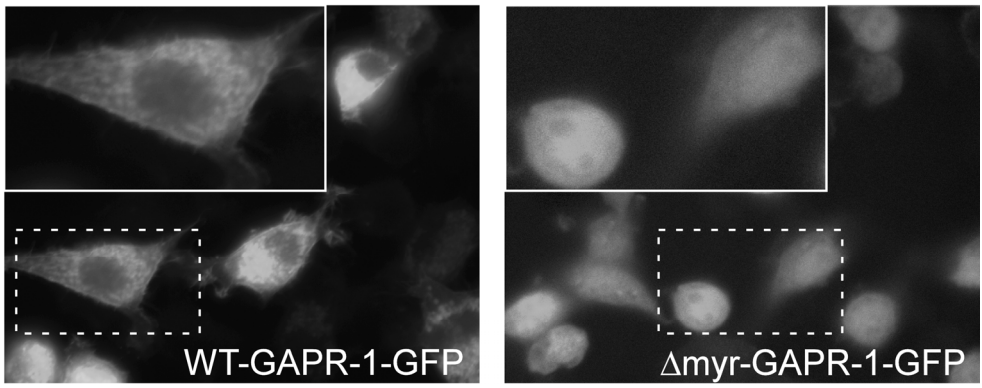
interaction and insertion of the myristoyl chain into the membrane. GAPR-1 is a basic protein at physiological pH, is myristoylated *in vivo* and is stably bound to membranes of the Golgi complex (Eberle et al., 2002). To investigate the contribution of the myristoyl moiety of GAPR-1 to membrane binding, the subcellular localization of non-myristoylated GAPR-1 in cells was compared to that of myristoylated GAPR-1. To this end, GAPR-1 was fluorescently tagged using GFP. Several attempts in this respect were made. N-terminal fusion of GFP to GAPR-1 will disrupt the myristoylation consensus sequence (see below) and cannot be used. C-terminal fusion of GFP to GAPR-1 leads to a severe mislocalization (data not shown). Therefore, a chimeric protein was generated of GFP that was fused into GAPR-1 between G125 and S126. When WT GAPR-1-GFP was expressed, it did not colocalize with Golgi markers (not shown). However, like endogenous GAPR-1 the mutant was clearly membrane bound, as fluorescence was detected on membranous structures (Fig. 3A). The fact that the protein was membrane-bound can be attributed to GAPR-1, as GFP by itself is cytosolic (data not shown). GAPR-1 contains a myristoylation consensus sequence at its N-terminus [Met-Gly-X-X-X-Ser/Thr, reviewed in (Farazi et al., 2001; Resh, 1999)]. To create non-myristoylated GAPR-1-GFP, the glycine was replaced by an alanine using site-directed mutagenesis. The non-myristoylated GAPR-1-GFP mutant, however, showed a clearly different subcellular localization, reminiscent of a partial localization to the cytosol (Fig. 3A).

To confirm that GAPR-1, in the absence of the myristoylation, has a partially cytosolic localization, cells were homogenized and the PNS was divided into a membrane fraction and a cytosolic fraction by ultracentrifugation. Both fractions were subjected to SDS-PAGE, after which the partitioning of GAPR-1 the different fractions was detected by Western blotting using an antibody to GAPR-1. Whereas WT GAPR-1 is exclusively found in the membrane fraction, the non-myristoylated GAPR-1 is partially (~ 30%) cytosolic (Fig. 3B). These results suggest that myristoylation *in vivo* may contribute to the stable membrane binding of GAPR-1, as non-myristoylated GAPR-1 shows less stable membrane localization.

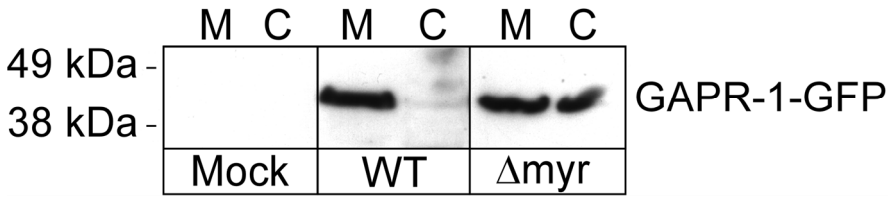
Myristoylation is not a requirement for raft partitioning of GAPR-1 in vivo

Double acylation of proteins may promote raft partitioning (Farazi et al., 2001; Zacharias et al., 2002). GAPR-1 participates in lipid-enriched membrane microdomains at the Golgi complex (Eberle et al., 2002). However, GAPR-1 is only myristoylated and it is not known whether a single acylation also contributes to the partitioning of proteins into rafts.

A



B



C

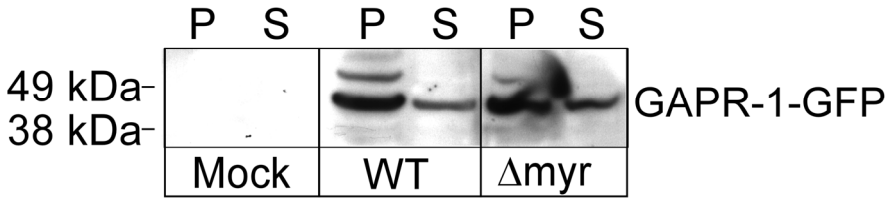


Figure 3: Non-myristoylated GAPR-1 partially localizes to the cytosol.
(A) Fluorescence microscopy on HeLa cells transfected with with WT GAPR-1-GFP (left panel) and non-myristoylated (Δ-myr)-GAPR-1-GFP (right panel). The insert shows the indicated area (dashed rectangle) at a higher magnification (B) HeLa cells were (mock)transfected with the indicated constructs (WT, Δ-myr). The transfected cells were homogenized, PNS was isolated and subsequently separated in a membrane pellet (M) and a cytosolic fraction (C) by ultracentrifugation. Proteins in the fractions resolved by SDS-PAGE and subsequently blotted for the presence of GAPR-1-GFP. The blots were probed with antibodies against GAPR-1.
(C) Membrane pellets of (B) were solubilized in 1% Triton X-100 for 30 minutes at 4 °C and separated in a detergent insoluble membrane pellet (P) and a detergent soluble supernatant (S)

Therefore, the role of the myristoylation in raft partitioning of GAPR-1 was investigated. GAPR-1 isolated from cells can be recovered in detergent-resistant membranes (Gkantiragas et al., 2001) and this property (insolubility in Triton X-100) was used as a measure of raft partitioning. Using PI-containing liposomes, non-myristoylated GAPR-1 is not enriched on detergent-insoluble membranes (DRMs) (not shown). To investigate the partitioning of myristoylated GAPR-1 to detergent-resistent membranes, we generated recombinant myristoylated GAPR-1 was generated. This could only be solubilized in the presence of detergent. Myristoylated GAPR-1 was incorporated into liposomes by detergent dilution using gel filtration, resulting in the generation of proteoliposomes (Materials and Methods). To discriminate myristoylated GAPR-1 from non-myristoylated GAPR-1, radio-labeled myristate was used. After incubation, 1% Triton X-100 was added to the proteoliposomes and DRMs were isolated by flotation on a sucrose gradient. Phospholipids were quantified by phosphate measurement and GAPR-1 was quantified by radioactivity. Compared to the starting material (not shown) and liposomes that were not treated with Triton X-100, 37% of the phosphate was recovered in the DRM fraction (Fig. 4C), indicating that approximately 1/3 of the lipids were present in lipid rafts. In contrast, all GAPR-1 could be recovered from the DRMs (Fig. 4 A, B). This indicates that on artificial membranes, myristoylation of GAPR-1 induces raft partitioning.

To investigate whether myristoylation leads to DRM localization of GAPR-1 *in vivo*, GFP-labeled myristoylated and non-myristoylated GAPR-1 were expressed in HeLa cells. Cells were then homogenized and a membrane pellet was made by centrifugation. The membranes were incubated with Triton X-100 and detergent-insoluble membranes were isolated by ultracentrifugation. DRMs and detergent-soluble membranes were resolved by SDS-PAGE and were blotted. The blots were then probed for GAPR-1 (Fig. 3C). When GAPR-1 is not myristoylated, a remarkably large fraction localizes to DRMs. When compared to non-myristoylated GAPR-1, myristoylated GAPR-1 did not show increased DRM localization. This may indicate that *in vivo*, other interactions (protein-protein, protein-lipid) are sufficient for raft partitioning and that myristoylation is not a requirement for raft localization of GAPR-1.

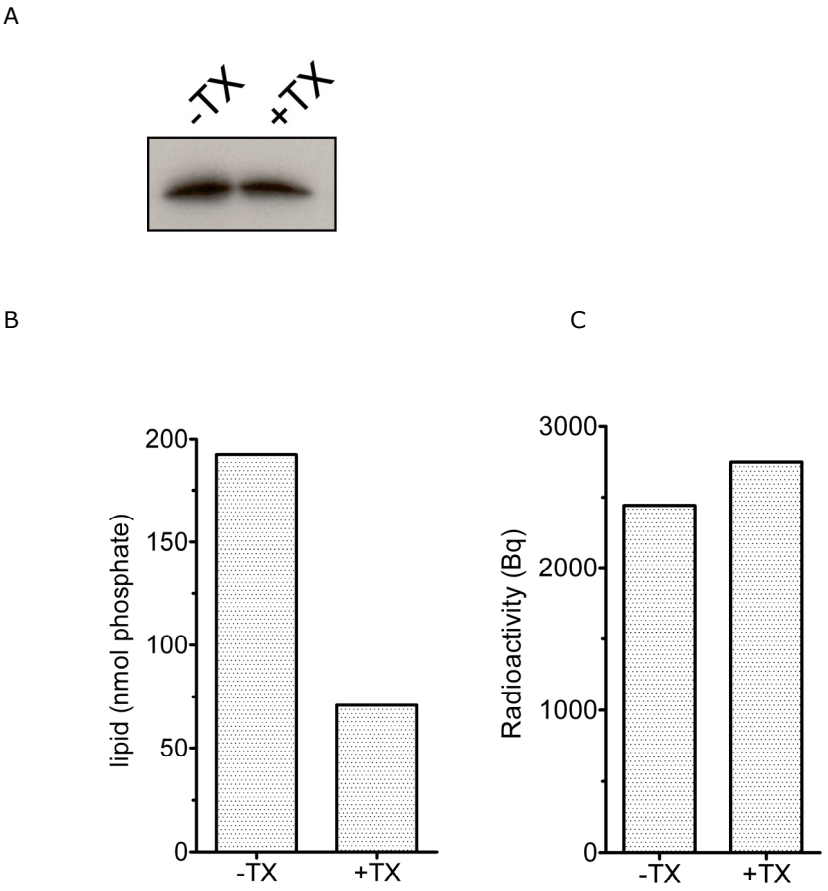
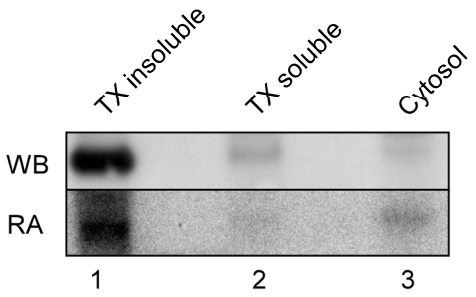


Figure 4 Myristoylated GAPR-1 participates in rafts of model membranes.
(A) Proteoliposomes containing myristoylated GAPR-1 (Materials and Methods) were incubated in the absence or presence of Triton X-100 (1%) for 30 minutes at 4°C. Total liposome membranes [left lane, in the absence of Triton X-100 (-TX)] and DRMs [right lane, in the presence of Triton X-100 (+TX)] were recovered by flotation on a sucrose gradient. Proteins in the top fraction of the gradient containing the lipids were precipitated, resolved by SDS-PAGE and analyzed by autoradiography.
(B) Quantitation of the presence of [^{3}H]myristate in the floated protein fraction. Radioactivity in 75 µl of the floated fraction was quantified by liquid-scintillation counting.
(C) Quantification of floated lipids by a phosphate measurement. Phosphate was measured according to Rouser (Materials and Methods) using 75 µl of the floated fraction. All lipids in the incubations were recovered in the floated fraction, as indicated by phosphate determinations of the input material versus floated liposomes in the absence of Triton X-100 (data not shown).

GAPR-1 is phosphorylated in vivo

The partitioning of myristoylated proteins to cellular membranes can be sensitive to posttranslational modifications that control the reversible translocation of myristoylated proteins onto membranes. The primary structure of GAPR-1 shows several putative phosphorylation sites as predicted by Netphos and Scansite (Table 1). To investigate whether GAPR-1 can be phosphorylated *in vivo*, CHO cells were incubated 4 h with radiolabeled inorganic phosphate ($^{32}P_i$). Immunoprecipitation with an antibody against GAPR-1 (Eberle et al., 2002) showed that the immunoprecipitated GAPR-1 is radioactively labeled due to incorporation of $^{32}P_i$ in the protein (data not shown). The extent of GAPR-1 phosphorylation was not affected by treatment with phosphatase inhibitors (data not shown). To analyze the possibility that phosphorylation changes the interaction of GAPR-1 with membranes a total cell membrane fraction was obtained from $^{32}P_i$-treated CHO cells. The membranes were resuspended in cold PEN buffer containing 1% Triton X-100 and detergent-soluble and insoluble fractions were analyzed for the presence of phosphorylated GAPR-1. A major pool of GAPR-1 is insoluble in Triton X-100 (Fig. 5A, lane 1, upper panel), and this fraction is phosphorylated (Fig. 5A, lane 1, bottom panel); a minor pool of GAPR-1 is soluble in Triton X-100 (Fig. 5A, lane 2, upper panel). This soluble fraction shows a low level of phosphorylation (Fig. 5A, lane 2, bottom panel). Interestingly, CHO cytosol of treated cells shows a barely detectable fraction of GAPR-1 by western blot analysis (Fig. 5A, lane 3, upper panel), but a relatively high level of phosphorylation is observed in this sample (Fig. 5A, lane 3, bottom panel). Quantitation of the radioactive signals shows that approximately 25% (calculated as percentage of total radioactive signal) of the incorporated phosphate in GAPR-1 has a cytosolic localization (Fig. 5B). As a result, the ratio of radioactivity : protein (3.1) is drastically increased in the cytosolic fraction (Fig. 5B). The soluble fraction in Triton X-100 represents approximately 10% of total radioactivity with no increase in the radioactivity : protein ratio (0.7). Most of the GAPR-1 is recovered in the detergent insoluble fraction with a radioactivity : protein ratio of 0.8. This indicates that there is no difference in GAPR-1 phosphorylation between different pools in the membrane, *i.e.* raft and non-raft fraction. These data suggest that GAPR-1 can be phosphorylated *in vivo* and that phosphorylation of GAPR-1 can play a role on the dynamics of GAPR-1 association with Golgi membranes.

A



B

	TX insoluble	TX soluble	Cytosol
WB	77	15	8
RA	65	10	25
Ratio RA/WB	0.8	0.7	3.1

Figure 5. Phosphorylation of GAPR-1 and partitioning into lipid rafts.
(A) Confluent CHO wt cells were incubated for 4 h in DMEM (phosphate and serum free) at 37 °C in the presence of [^{32}P] 0,25 mCi/ml. Cells were washed, harvested and homogenized. The homogenate was centrifuged 1 h at 100,000 x g to isolate a total membrane fraction (pellet) and a cytosol fraction (supernatant). The total membrane fraction was dissolved in 1% Triton X-100/PEN buffer and incubated for 30 min at 4 °C. After incubation, the fraction was centrifuged 1 h at 100,000 x g to yield a soluble (lane 2) and an insoluble (lane 1) fraction. GAPR-1 was immunoprecipitated from both fractions as well as from cytosol of homogenized cells (lane 3). Proteins in the immunoprecipitates were separated by SDS-PAGE and analyzed for the presence of GAPR-1 by Western blotting (upper panel, WB) and for phosphorylation of GAPR-1 by autoradiography (lower panel, RA). (B) Quantification of band intensity of the Western blot shown in A and of the radioactive signals shown in A. Shown are percentages relative to the total signal.

Discussion

In the present study we investigated the effect of phosphoinositide binding, myristoylation, and phosphorylation on interaction of GAPR-1 with membranes.

GAPR-1 binding to phosphoinositides

Phosphoinositides are known to function as signaling lipids. High concentrations of specific phosphoinositides can be locally generated by phosphorylation of phosphatidylinositol and phosphoinositides or dephosphorylation of phospho-

inositides. As a result, phosphoinositides attract proteins to specific membranes [reviewed by (De Matteis et al., 2002; De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006)]. On the Golgi, PI(4)P is the predominant phosphoinositide (De Matteis et al., 2002; Di Paolo and De Camilli, 2006; Wang et al., 2003), whereas on exosomes and late endosomes PI(3)P and PI(3)P derivatives prevail (Gillooly et al., 2000). GAPR-1 showed affinity for these lipids *in vitro* and it is temping to speculate that these interactions are involved in the localization of GAPR-1 to the Golgi complex (Eberle et al., 2002) and exosomes (Kaloyanova, unpublished) ,

GAPR-1 has a strong affinity for phosphoinositides with a rather promiscuous phosphoinositide binding pattern. In liposomes, GAPR-1 shows a preference for bisphosphates instead of monophosphates, which is in contrast to the protein lipid overlay in which GAPR-1 preferred monophosphates over bisphosphates. The higher affinity of GAPR-1 for bisphosphates in the liposome binding assay may indicate that electrostatics play an important role. However, a strong negative charge is not the sole binding criterion for GAPR-1, as the affinity for the most negatively charged lipid, PI(3,4,5)P_3, is relatively low in both the protein-lipid overlay and the liposome-binding experiment.

GAPR-1 lipid interactions

A number of differences were observed in the binding of GAPR-1 to lipids when comparing the liposome binding assay with the nitrocellulose overlay assay. There may be important differences in lipid presentation to GAPR-1 on nitrocellulose membranes as compared to lipid presentation in liposomes. These differences may provide a clue to the mechanism of lipid recognition of GAPR-1. First, lipid packing and ordering will differ between the two conditions. On the PIP Strip, pure lipid is spotted, whereas in liposomes the phosphoinositides are diluted in PC, a bulk lipid for which GAPR-1 has virtually no affinity. In addition, whereas the lipids in liposomes have a bilayer ordering, it is not known how lipids are oriented on a nitrocellulose membrane. Second, membrane dynamics are different between the two conditions as lipids on the nitrocellulose membrane are fixed, whereas lipids in liposomes may freely move and diffuse. Third, GAPR-1 was incubated in the overlay assay at room temperature, whereas it was incubated at 37 °C in the liposome binding assay. It was previously shown that GAPR-1 binding to PI is significantly lower at room temperature than at 37 °C (van Galen et al., 2008). Fourth, the experiments were performed in different buffers. For the protein lipid overlay, Tween 20 is included in the buffers to reduce background signals. Although Tween 20 is a mild detergent, it

may interfere with the lipid binding of GAPR-1. Fifth, protein concentration and protein/lipid ratios differ between the two methods. For the overlay and liposome binding experiments, GAPR-1 concentrations of 0.45 µg/ml and 0.22 mg/ml were used, respectively. Dimer formation may be a regulatory mechanism of GAPR-1 function (Serrano et al., 2004) which may involve membrane binding (Chapter 3). As the concentration of GAPR-1 in the liposome binding assay is ~500 times higher, it is possible that under these conditions more dimers are present, affecting phosphoinositide specificity of GAPR-1. In summary, although there are noticeable differences between the two methods, they both point out that GAPR-1 binds phosphoinositides.

Mechanism(s) of phosphoinositide binding

Several proteins contain domains such as the pleckstrin homology domain and the FYVE domain for specific binding to phosphoinositides (Lemmon, 2008; Stenmark et al., 2002). Other proteins interact with phosphoinositides via the surface of the protein rather than a lipid binding pocket (Overduin et al., 2001). This possibly enables proteins to bind phosphoinositides with a low specificity as there is no necessity for the headgroups to fit exactly in a binding pocket. The promiscuous binding pattern of GAPR-1 to phosphoinositides is reminiscent of that of A-Raf and Raf-1 (Johnson et al., 2005). The phosphoinositide binding of A-Raf and Raf-1 is also promiscuous, but their activity can be regulated by phosphoinositides and therefore the phosphoinositide binding may be physiologically relevant. Also the MARCKS proteins bind phosphoinositides in a promiscuous way and lack a specific phosphoinositide binding domain (McLaughlin et al., 2002). However, MARCKS proteins bind/sequester $PI(4,5)P_2$ with relatively high affinity, even when PS is present in 300 fold excess (Gambhir et al., 2004). The role of GAPR-1 binding to phosphoinositides remains to be determined. One possibility is that GAPR-1 undergoes a conformational change upon phosphoinositide binding. For instance, GAPR-1 dimers change their conformation upon binding to phytic acid, which is an inositol polyphosphate resembling phosphoinositide headgroups (Chapter 3).

Membrane and raft localization of GAPR-1

Several interactions may contribute to the membrane and raft localization of GAPR-1. Here we have studied the effects of myristoylation, protein-lipid interactions, and phosphorylation. Protein-protein interactions were not the scope of this study but may be involved as well.

The strong membrane binding of myristoylated GAPR-1 *in vivo* raises the question how membrane binding of GAPR-1 is regulated and whether it can be released from the membranes at all. In order to be released from membranes, strong electrostatic interactions must be disrupted and the myristoyl moiety must be shielded from an aqueous environment. Different strategies have been devised to solve these issues for myristoylated proteins. MARCKS proteins attach very strongly to membranes by binding to negatively charged lipids and by a myristoyl anchorage, but are released from membranes by neutralization of their positive lipid binding domain by phosphorylation (Kim et al., 1994). The small GTP-binding protein Arf1 buries its myristate moiety in a hydrophobic protein cavity upon binding of GDP, resulting in a soluble protein. Upon binding of GTP, a conformational change is induced, resulting in exposure of the myristoyl moiety and its insertion in the membrane (Antonny et al., 1997). Which of those mechanisms apply to GAPR-1 and whether a myristoyl-switch is involved in GAPR-1 remains to be investigated. We have been able to detect small amounts of GAPR-1 in cytosol upon phosphorylation. This behaviour is different from that of other myristoylated proteins, such as ARF and MARCKS, which show dramatic cytosolic relocalization upon the appropriate signal. Possibly GAPR-1 cannot effectively shield the myristoyl-moiety by itself, as may be indicated by the fact that myristoylated GAPR-1 is not soluble, unlike other myristoylated proteins. This would imply the necessity of cytosolic co-factors that bind GAPR-1 to shield the myristate group from an aqueous environment.

Myristoylation in combination with palmitoylation may promote raft partitioning (Farazi et al., 2001). Whereas palmitoylation is a dynamic modification, myristoylation is a permanent cotranslational or posttranslational modification. GAPR-1 is myristoylated, but there are currently no indications that GAPR-1 is also palmitoylated. Myristoylation by itself is not sufficient to promote raft participation. *In vivo*, GAPR-1 is localized on membrane microdomains at the Golgi complex (Gkantiragas et al., 2001). The liposome binding experiment suggested that myristoylation may give GAPR-1 biophysical properties that promote partitioning of GAPR-1 into DRMs *in vitro*. However, no difference in DRM partitioning was found between myristoylated and non-myristoylated GAPR-1 *in vivo.* Therefore, specific protein or lipid binding interactions are involved in the partitioning of GAPR-1 to DRMs. One possible candidate is caveolin-1, a protein known to interact with GAPR-1 (Eberle et al., 2002).

GAPR-1 membrane binding in the cell is thus potentially regulated by several factors, including myristoylation, a GAPR-1-Caveolin-1 interaction (Eberle et al., 2002), a coiled-coil domain allowing additional protein-protein interactions (Eberle et al., 2002), phosphorylation, and protein-lipid interactions including binding to PI and phosphoinositides. The multitude of interactions may explain why GAPR-1, in contrast to many other myristoylated proteins, cannot be recovered in significant amounts from the cytosolic fraction. It is tempting to speculate that the myristoyl-moiety of GAPR-1 is not involved in establishing an equilibrium in localization between membranes and cytosol, but in establishing an equilibrium in protein-protein interactions, either between GAPR-1 and other proteins, or between the monomer and dimer configuration of GAPR-1 on the membrane.

It is remarkable that a relatively small protein may be involved in so many interactions, but this may explain the difficulty in tagging GAPR-1 with e.g. GFP. Tags have been inserted at several positions, but so far we have been unsuccessful in creating a tagged GAPR-1 construct that is correctly localized (unpublished). Many constructs show different phenotypes, indicating that, depending on the insertion of the tag, different interactions are perturbed. Here we showed a GFP-tagged GAPR-1 construct with a relatively mild phenotype. Systematic screening of the different phenotypes may allow a structure-function analysis by mapping of the various interactions of GAPR-1 to the surface of the protein.

Acknowledgements

We thank Ger Arkesteijn for help with the FACS analysis and Coos Batenburg for critically reviewing our manuscript. This work was supported by a grant from the Research Council for Earth and life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO).

References

Adachi, J., C. Kumar, Y. Zhang, J.V. Olsen, and M. Mann. 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biol*. 7:R80.

Antonny, B., S. Beraud-Dufour, P. Chardin, and M. Chabre. 1997. N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry*. 36:4675-84.

Baxter, R.M., T.P. Crowell, J.A. George, M.E. Getman, and H. Gardner. 2006. The plant pathogenesis related protein GLIPR-2 is highly expressed in fibrotic kidney and promotes epithelial to mesenchymal transition in vitro. *Matrix Biol*.

Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 37:911-7.

Brown, D.A., and E. London. 1997. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem Biophys Res Commun*. 240:1-7.

Brown, D.A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol*. 14:111-136.

Chen, R.F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J Biol Chem*. 242:173-81.

De Matteis, M., A. Godi, and D. Corda. 2002. Phosphoinositides and the golgi complex. *Curr Opin Cell Biol*. 14:434-447.

De Matteis, M.A., and A. Godi. 2004. PI-loting membrane traffic. *Nat Cell Biol*. 6:487-92.

Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature*. 443:651-7.

Duronio, R.J., E. Jackson-Machelski, R.O. Heuckeroth, P.O. Olins, C.S. Devine, W. Yonemoto, L.W. Slice, S.S. Taylor, and J.I. Gordon. 1990. Protein N-myristoylation in Escherichia coli: reconstitution of a eukaryotic protein modification in bacteria. *Proc Natl Acad Sci U S A*. 87:1506-1510.

Eberle, H.B., R.L. Serrano, J. Fullekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, and J.B. Helms. 2002. Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex. *J Cell Sci*. 115:827-838.

Eisenberg, I., M. Barash, T. Kahan, and S. Mitrani-Rosenbaum. 2002. Cloning and characterization of a human novel gene C9orf19 encoding a conserved putative protein with an SCP-like extracellular protein domain. *Gene*. 293:141-148.

Farazi, T.A., G. Waksman, and J.I. Gordon. 2001. The biology and enzymology of protein N-myristoylation. *J Biol Chem*. 276:39501-4.

Gambhir, A., G. Hangyas-Mihalyne, I. Zaitseva, D.S. Cafiso, J. Wang, D. Murray, S.N. Pentyala, S.O. Smith, and S. McLaughlin. 2004. Electrostatic sequestration of PIP2 on phospholipid membranes by basic/aromatic regions of proteins. *Biophys J*. 86:2188-207.

Gillooly, D.J., I.C. Morrow, M. Lindsay, R. Gould, N.J. Bryant, J.M. Gaullier, R.G. Parton, and H. Stenmark. 2000. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *Embo J*. 19:4577-88.

Gkantiragas, I., B. Brugger, E. Stuven, D. Kaloyanova, X.Y. Li, K. Lohr, F. Lottspeich, F.T. Wieland, and J.B. Helms. 2001. Sphingomyelin-enriched microdomains at the Golgi complex. *Mol Biol Cell*. 12:1819-1833.

Groves, M.R., A. Kuhn, A. Hendricks, S. Radke, R.L. Serrano, J.B. Helms, and I. Sinning. 2004. Crystallization of a Golgi-associated PR-1-related protein (GAPR-1) that localizes to lipid-enriched microdomains. *Acta Crystallogr D Biol Crystallogr*. 60:730-732.

Johnson, L.M., K.M. James, M.D. Chamberlain, and D.H. Anderson. 2005. Identification of key residues in the A-Raf kinase important for phosphoinositide lipid binding specificity. *Biochemistry*. 44:3432-3440.

Kim, J., T. Shishido, X. Jiang, A. Aderem, and S. McLaughlin. 1994. Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles. *J Biol Chem*. 269:28214-9.

Lemmon, M.A. 2008. Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol*. 9:99-111.

McLaughlin, S., J. Wang, A. Gambhir, and D. Murray. 2002. PIP(2) and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct*. 31:151-75.

Overduin, M., M.L. Cheever, and T.G. Kutateladze. 2001. Signaling with phosphoinositides: better than binary. *Mol Interv*. 1:150-9.

Peitzsch, R.M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry*. 32:10436-43.

Pisitkun, T., R.F. Shen, and M.A. Knepper. 2004. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 101:13368-73.

Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta*. 1451:1-16.

Rouser, G., S. Fkeischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*. 5:494-6.

Serrano, R.L., A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, and M.R. Groves. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. *J Mol Biol*. 339:173-83.

Stenmark, H., R. Aasland, and P.C. Driscoll. 2002. The phosphatidylinositol 3-phosphate-binding FYVE finger. *FEBS Lett*. 513:77-84.

Stoorvogel, W., M.J. Kleijmeer, H.J. Geuze, and G. Raposo. 2002. The biogenesis and functions of exosomes. *Traffic*. 3:321-330.

Taniguchi, H. 1999. Protein myristoylation in protein-lipid and protein-protein interactions. *Biophys Chem*. 82:129-37.

van Galen, J., B. van Balkom, E. Stueven, and J.B. Helms. 2008. Strong binding of GAPR-1 to phosphatidylinositol: Indications for a novel membrane anchor. *Submitted*.

van Loon, L.C., M. Rep, and C.M. Pieterse. 2006. Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol*. 44:135-62.

Wang, Y.J., J. Wang, H.Q. Sun, M. Martinez, Y.X. Sun, E. Macia, T. Kirchhausen, J.P. Albanesi, M.G. Roth, and H.L. Yin. 2003. Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell*. 114:299-310.

Zacharias, D.A., J.D. Violin, A.C. Newton, and R.Y. Tsien. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science*. 296:913-6.

Chapter 5

Summarizing Discussion

Multiple factors contribute to membrane binding of GAPR-1

Golgi-Associated Plant Pathogenesis-Related protein 1 (GAPR-1) is a mammalian protein that is well conserved among vertebrates and predominantly expressed in immunocompetent cells. GAPR-1 shares the highest homology with pathogenesis related proteins group 1 (PR-1) in plants (Eberle et al., 2002). In contrast to PR-1 proteins, GAPR-1 is not secreted but stably bound to the cytosolic leaflet of intracellular membranes. In resting cells, GAPR-1 localizes to lipid rafts at the Golgi complex (Eberle et al., 2002). During phagocytosis, some GAPR-1 relocalizes to phagosomes (Kaloyanova et al., unpublished data)(Burlak et al., 2006). Two proteomic studies (Adachi et al., 2006; Pisitkun et al., 2004) and unpublished data (Kaloyanova et al.) show that GAPR-1 also localizes to exosomes. So far, the function of GAPR-1 is unknown. Elucidation of the membrane-binding characteristics will provide insights into potential regulatory mechanisms of the function of GAPR-1. There are several factors contributing to membrane localization of GAPR-1. In this study we investigated the role of GAPR-1 binding to negatively charged lipids, and modifications of the GAPR-1 protein including myristoylation and phosphorylation. The results obtained in our studies suggest that binding of GAPR-1 to membranes is multi-factorial, which allows regulation at multiple levels.

Binding of GAPR-1 to negatively charged lipids

The calculated isoelectric point of GAPR-1 is 9.4 (Eberle et al., 2002) and therefore GAPR-1 is predicted to be positively charged at physiological pH. The charge is concentrated at one area of the GAPR-1 surface (Serrano et al., 2004), which may facilitate binding of GAPR-1 to negatively charged lipids at membranes. Data in **chapter 2** show that non-myristoylated GAPR-1 can indeed bind to membranes containing negatively charged lipids. Binding of GAPR-1 to membranes correlates with the concentration of negatively charged lipids in those membranes. Therefore, one factor contributing to the overall affinity of GAPR-1 for biological membranes *in vivo* may be a strong affinity for membranes containing a high level of negatively charged lipids. In **chapter 2** it was shown that GAPR-1 can bind to all tested negatively charged lipids with a single negative charge. GAPR-1 has a higher affinity for phosphatidic acid (PA), which may be (partially) explained by the fact that this lipid has a slightly higher charge at neutral pH (Marsh, 1990). Phosphatidylserine and phosphatidylinositol (PI) are the most abundant negatively charged lipids in biological membranes (Ansell, 1973) and they may contribute the most to the electrostatic interaction of GAPR-1 with lipids in membranes. It cannot be excluded, however, that

high concentrations of e.g. PA occur at defined areas or domains of the membrane and that this becomes a significant factor in GAPR-1 binding to biological membranes.

In **chapter 4** we showed that GAPR-1 has a high affinity for phosphoinositides. Interestingly, binding of GAPR-1 to PI(4,5)P_2 is stronger than to PI(3,4,5)P_3, the latter of which is more negatively charged. This implies that in addition to charge, GAPR-1 recognizes other features of this lipid class. Phosphoinositides *in vivo* are present in low amounts in cellular membranes. However, during cellular processes involving membrane dynamics, high concentrations of phosphoinositides can be generated locally. By generation of phosphoinositides in membranes, cytosolic proteins with specific phosphoinositide binding domains are recruited to the organelle. During phagocytosis and receptor mediated endocytosis, for instance, signaling by phosphoinositides plays an essential role (Yeung et al., 2006). Cells generate defined phosphoinositides at particular organelles in such a precise way that these lipids are one of the factors contributing to the identity of an organelle (Munro, 2002). Binding to phosphoinositides could represent an attractive mechanism contributing to the membrane characteristics of GAPR-1. Clearly it is not the sole membrane-localization factor, as GAPR-1 also binds to PI(4,5)P_2, which localizes *in vivo* to the plasma membrane, whereas GAPR-1 mainly localizes to the Golgi complex. The binding to phosphoinositides may also allow the regulation of GAPR-1 by an inositol phosphate switch, which will be discussed in more detail below (see *Dimerization of GAPR-1*).

GAPR-1 binding to phosphatidylinositol

During screening for binding of GAPR-1 to negatively charged lipids (**chapter 2**), it appeared that GAPR-1 bound unusually strong to PI. The PI binding of GAPR-1 was resistant to extraction with organic solvents, boiling under reducing conditions prior to SDS-PAGE analysis, and the binding remained intact during mass spectrometric analysis. The binding to PI resulted in membrane attachment of GAPR-1. There are some examples of other proteins known to be modified with a phospholipid. Atg8 is modified by Atg3 with phosphatidylethanolamine (PE), which leads to a conformational change of Atg8 and to localization to membranes (Ichimura et al., 2004). Atg8 plays a role in autophagocytosis and linkage to PE is part of the signaling mechanism. Lipidation may thus act as a molecular switch. Another phospholipid linkage is reported for ubiquitin during infection of cells by baculoviruses (Guarino et al., 1995). By lipidation, ubiquitin is localized to the cytosolic leaflet of the virion.

This may be a mechanism for the virus to capture ubiquitin and possibly other proteins as well on the cytosolic leaflet of the viral membrane. In the examples described above, lipid binding leads to membrane localization of a peripheral protein. The binding of PI to GAPR-1 may serve the same purpose. Modification by PI may be the equivalent of a glycosylphosphatidylinositol (GPI)-anchor albeit at the cytosolic leaflet. However, whereas GPI-anchored proteins partition to lipid rafts, PI-anchoring of GAPR-1 does not seem to affect raft participation as judged by the localization of GAPR-1 to detergent-resistant membranes in the absence and presence of bound PI. This suggests that PI binding has no influence on raft localization of GAPR-1.

Myristoylation of GAPR-1

GAPR-1 *in vivo* is myristoylated, which is a co-translational modification that may enhance or stabilize the interaction of GAPR-1 with membranes. The contribution of myristoylation to the membrane binding characteristics of GAPR-1 was investigated in **chapter 4.** Mutation of the consensus sequence for myristoylation of GAPR-1 resulted in the generation of a non-myristoylated GAPR-1 protein. This GAPR-1 mutant was partially (30%) localized to the cytosol. In contrast, virtually all wild type GAPR-1 was membrane bound. These results indicate that myristoylation contributes to membrane binding of GAPR-1. Dual acylation can lead to raft localization of proteins (Farazi et al., 2001; Zacharias et al., 2002). For GAPR-1, only a single acyl modification has been described. Although myristoylation of GAPR-1 resulted in participation to detergent-resistant membranes *in vitro*, there was no difference between myristoylated and non-myristoylated GAPR-1 in participation to detergent-resistant membranes *in vivo*. Therefore, myristoylation contributes to membrane binding of GAPR-1, but probably not to raft partitioning. Other factors such as protein-protein interactions may be involved in raft partitioning. For example, GAPR-1 binds to caveolin-1 (Eberle et al., 2002; Okamoto et al., 1998). Whereas the majority of caveolin-1 is localized to the plasma membrane, caveolin-1 has been found at the trans-Golgi network and at earlier compartments (Fielding and Fielding, 2003; Gkantiragas et al., 2001). By binding to caveolin-1 at the Golgi complex, membrane binding of GAPR-1 may be stabilized. Factors involved in raft partitioning of GAPR-1 will be further discussed below (see *Raft partitioning of GAPR-1*).

Membrane binding of GAPR-1 may be regulated by phosphorylation

As described above, GAPR-1 has several characteristics that may promote its localization to biological membranes: a positively charged protein surface area, an

interaction with a membrane-resident protein, a myristoyl anchor and a phosphatidylinositol anchor. According to the two-signal model of M. Resh (Resh, 1999), two of such interactions are sufficient for stable membrane localization. Indeed, GAPR-1 is tightly bound to biological membranes as salt-stripping of isolated membranes or treatment of cells with Brefeldin A, which causes a redistribution of GAPR-1 in cells, does not release GAPR-1 from membranes (Eberle et al., 2002). However, a minor amount of GAPR-1 does localize to the cytosol, which indicates that under some circumstances GAPR-1 can be released from biological membranes. In **chapter 4**, metabolic labeling with radioactive phosphate shows that the cytosolic pool of GAPR-1 is highly phosphorylated relative to the membrane-bound fraction. By phosphorylation, negative charge is added to GAPR-1, which could reduce its affinity for negatively charged membrane lipids. GAPR-1 bears multiple putative phosphorylation sites (Table 1), but it is not known whether GAPR-1 is in fact phosphorylated at multiple sites. Reduction of membrane affinity by phosphorylation has been described for MARCKS proteins (McLaughlin and Aderem, 1995).

Netphos

Position	site
48	EALA**S**TRIL
55	ILKH**S**PESS
58	HSPE**S**SRGQ
72	AWAS**Y**DQTG
75	SYDQ**T**GKEV
85	DRWY**S**EIKN
112	VWKN**T**KKMG
121	VGKA**S**ASDG
123	KASA**S**DGSS

Scansite

Position	site
126	ASDG**S**SFVV

Table 1 Putative phosphorylation sites in GAPR-1.
Phosphorylation sites were predicted by analysis of the amino acid sequence of GAPR-1 by Netphos 2.0 [http://www.cbs.dtu.dk/services/NetPhos/, (Blom et al., 1999)] and Scansite [http://scansite.mit.edu/ (Obenauer et al., 2003)]. In the table only the sites are shown that score above the default probability threshold of the websites. Residues that are predicted to be phosphorylated are shown in bold.

MARCKS proteins have a basic effector domain that is relatively unstructured (McLaughlin et al., 2002). The phosphorylation sites lie within this basic domain. Abrogation of the electrostatic interaction with the membrane by phosphorylation results in translocation of MARCKS from membranes to cytosol. This mechanism is referred to as the "myristoyl/electrostatic switch". In the predicted membrane orientation of GAPR-1 (shown in **chapter 1**), most of the potential phosphorylation sites (serines, threonines and tyrosines) are oriented to the cytosol and are not localized within the basic (positively charged) surface area of GAPR-1.

Release of GAPR-1 from the membrane into the cytosol requires shielding of its myristoyl moiety from an aqueous environment. Other permanently acylated proteins have devised various strategies to protect the acyl group. Rab proteins bind to membranes via one or two prenyl groups (Pfeffer and Aivazian, 2004). Upon inactivation of Rab proteins, cytosolic Rab GDP dissociation inhibitor (GDI) proteins can shield the hydrophobic prenyl groups from the cytosol, and aid the release of Rab proteins from the membrane. Phosphorylation may also serve to enable GAPR-1 to bind cytosolic proteins and thus compete for factors that are involved in membrane binding, resulting in release of GAPR-1 from the membrane. Alternatively, phosphorylation may lead to a conformational change of GAPR-1, which enables the release from the membrane. For Arf1 was shown that it can undergo a conformational change, which allows to bury its myristate group in a hydrophobic cleft on the protein surface (Antonny et al., 1997). This mechanism is referred to as a "myristoyl-conformational switch" (Farazi et al., 2001). It remains to be established whether phosphorylated GAPR-1 is released from the membrane by a similar mechanism, as an obvious hydrophobic cleft has not yet been identified in the GAPR-1 structure.

Dimerization of GAPR-1

In **chapter 3** it is shown that phytic acid induces dimerization of GAPR-1 and that it inhibits membrane binding of GAPR-1. Interestingly, dimerization itself does not inhibit membrane binding of GAPR-1 as a Δ4 mutant of GAPR-1, which has a strong tendency to form dimers (Serrano et al., 2004), showed enhanced membrane binding characteristics *in vitro*. The detection of GAPR-1 dimers on Golgi membranes *in vivo* (Eberle et al., 2002) strengthens our conclusion that dimer formation is not inhibitory for membrane binding. In the presence of phytic acid, however, GAPR-1 crystallizes in a different conformation with a rotation of the GAPR-1 monomers relative to each other in the GAPR-1 dimer. This rotation may be responsible for the observed

inhibition of membrane binding of GAPR-1 in the presence of phytic acid. The cellular concentration of phytic acid is in close proximity of the concentration that shows half maximal inhibition of GAPR-1 binding to liposomes *in vitro*. Therefore, phytic acid may also regulate membrane binding of GAPR-1 *in vivo*. It is also possible that not phytic acid itself, but structurally related molecules regulate membrane binding of GAPR-1. Based on the similarity of the head groups of phosphoinositides to phytic acid, it is tempting to speculate that these lipids are not only involved in the membrane binding of GAPR-1, but also in the regulation of GAPR-1 dynamics. GAPR-1 shows promiscuous binding to phosphoinositides (**chapter 4**), but some degree of specificity is observed as *e.g.* GAPR-1 shows less binding to PI(3,4,5)P_3 as compared to PI(4,5P)$_2$. Thus, by changing the phosphoinositide content of membranes, GAPR-1 binding and dynamics may be affected. This suggests a regulation by an inositol phosphate-mediated switch, similar to what has been observed for other proteins (Datta et al., 2007; Milano et al., 2006).

Raft partitioning of GAPR-1

As discussed above, there are many factors that promote or inhibit membrane localization of GAPR-1. It is less clear, however, how raft localization of GAPR-1 is regulated. Myristoylation and PI binding did not result in enhanced DRM participation. Possibly, the interaction of GAPR-1 with caveolin-1 is important for its DRM participation as caveolin-1 is a well established raft-resident protein. This could be further investigated by use of caveolin-1 knockout cells or by use of a GAPR-1 mutant without the putative caveolin binding motif. Another mechanism to enter rafts may be dimerization or multimerization of GAPR-1. In **chapter 3** was shown that phytic acid affected dimerization of GAPR-1 by an intermolecular conformational change in the GAPR-1 dimer. Similarly, phosphoinositides, which contain an inositol phosphate moiety, may affect raft participation of GAPR-1 by affecting its dimerization properties. A change in protein conformation upon binding to phosphoinositides has been demonstrated for other proteins. For instance, WASP, a protein that is involved in the regulation of actin dynamics, changes its conformation upon binding to PI(4,5)P_2 (Prehoda et al., 2000). In the case of GAPR-1, a conformational change may lead to dimerization or multimerization, which may affect raft participation. Alternatively, binding to phosphoinostides that are enriched in lipid rafts may affect raft participation of GAPR-1.

Future studies on the function of GAPR-1

The Pfam database (http://pfam.sanger.ac.uk/) groups proteins according to their domain structure. In this database, GAPR-1 is a member of the family of Sperm Coating Protein (SCP) like proteins (accession number: PF00188). The general function of this protein family is poorly understood, as its members display very heterogeneous functions and SCP protein members have different cellular localizations. The function of the SCP domain may provide important indications for the function of GAPR-1, as the SCP domain covers more than 70% of the entire GAPR-1 molecule. The SCP-domain containing proteins helothermine (Morrissette et al., 1995) and Pseudechetoxin (Brown et al., 1999) have been implicated in ion channel inhibition. In these cases, the role of the SCP domain has not been further explored. The SCP-domain containing proteins Natrin and Ablomin in snake venoms were also found to block ion channels (Wang et al., 2006; Yamazaki et al., 2002). However, the SCP domain in these venoms was not involved in the block of the ion channel. Similarly**,** the SCP-domain containing protein Tpx-1 in humans is related to ion channel toxins and regulates ryanodine receptor Ca2+ signaling with its CRISP domain, but not with its SCP domain (Gibbs et al., 2006). Therefore, the SCP-domain may have an accessory function. Interestingly the Xenopus (frog) Xfeb protein consists of 5 SCP domains (Li et al., 2006). Possibly it is an adaptor protein which may act as a scaffold for protein-protein interactions.

One of the strongest suggestions so far for the function of the SCP domain is a proteolytic activity that has been described for Tex31 (Milne et al., 2003). This SCP-domain containing protein may use a putative catalytic triad, which is also present in GAPR-1 (Serrano et al., 2004). An alternative catalytic triad with serine protease activity has been suggested at the interface of the dimer of GAPR-1 (Serrano et al., 2004).

Since the widespread use of DNA microarrays, a wealth of information for gene expression is available and is still accumulating. In general, DNA arrays are used to identify genes that are up or down regulated during various conditions of a cell or to screen for genes which are expressed in a given tissue or cell type. However, these data can also be used to screen for genes that are coexpressed with the gene of interest. The coexpressed genes may give an indication of the process(es) in which the gene of interest is involved. For this purpose an online DNA microarray data database has been generated (http://bioinformatics.ubc.ca/Gemma/), which searches for coexpressed genes based on the method described in (Lee et al., 2004). It is of note that this website is still in its beta phase and therefore results should be

treated with caution. A search for coexpression of proteins with GAPR-1 revealed 133 and 339 genes in human (gene: c9orf19) and in mouse (gene: glipr2), respectively. Subsequently, these genes were grouped by a hypergeometric test, which detects the coverage of genes of a process among a given gene population [GATHER: http://gather.genome.duke.edu/ (Chang and Nevins, 2006)]. Gene annotations of processes that contain the highest coverage of genes within the annotation "Biological process" are shown in table 2. Although there are 22 subsections ("annotations" or "processes") possible for GO: biological process, only 4 significant subsections were found for human and 4 for mouse. Two subsections overlapped, resulting in a total of 6 different subsections of the main annotation "Biological process". Each subsection is further subdivided in smaller subsections or child annotations. When the coverage of co-expressed genes in a particular child annotation is high, this may indicate an involvement of GAPR-1 in this particular process as well. As coexpressed genes of GAPR-1 are found for the annotation "cellular communication" and "response to stimulus" in both organisms, it suggests that GAPR-1 may play a role in these processes. A role in "response to stimulus" is in agreement with our finding of GAPR-1 localization to phagosomes and a role in "cellular communication" is in agreement with the presence of GAPR-1 on exosomes. It will be interesting to study the dynamics of GAPR-1 in e.g. knockout cells of the genes present in each of these annotations. Exploration of the specific genes that are coexpressed with GAPR-1 may give important insights and suggestions for future investigations on GAPR-1 function.

Table 2. Gene ontology of coexpressed genes of GAPR-1 in human (c9orf19) and mouse (glipr-2).
Coexpressed genes of GAPR-1 were retrieved from http://bioinformatics.ubc.ca/Gemma/ and were subsequently annotated by gene ontology using GATHER http://gather.genome.duke.edu/ (Chang and Nevins, 2006)). The nine (human) and ten (mouse) gene annotations of processes that contain the highest coverage of genes within the annotation "biological process" are shown in bold. The gene ontology parent families which the annotations belong to are shown in normal type.

Gene ontology of genes coexpressed with GAPR-1 (c9orf19) in human

Gene_Ontology (GO:0003673)
> biological_process (GO:0008150)
- metabolic process (GO:0008152)
- cellular metabolic process (GO:0044237)
- primary metabolic process (GO:0044238)
- biological regulation (GO:0065007)
- regulation of biological process (GO:0050789)
- regulation of cellular process (GO:0050794)
- response to stimulus (GO:0050896)
- immune response (GO:0006955)
- defense response (GO:0006952)
- response to biotic stimulus (GO:0009607)
- response to other organism (GO:0051707)
- cellular process (GO:0009987)
- cell communication (GO:0007154)

Gene ontology of genes coexpressed with GAPR-1 (glipr-2) in mouse

Gene_Ontology (GO:0003673)
> biological_process (GO:0008150)
- multicellular organismal process (GO:0032501)
- multicellular organismal development (GO:0007275)
- system process (GO:0003008)
- neurological system process (GO:0050877)
- sensory perception (GO:0007600)
- response to stimulus (GO:0050896)
- response to external stimulus (GO:0009605)
- detection of external stimulus (GO:0009581)
- cellular process (GO:0009987)
- cell adhesion (GO:0007155)
- cell communication (GO:0007154)
- signal transduction (GO:0007165)
- intracellular signaling cascade (GO:0007242)
- cell surface receptor linked signal transduction (GO:0007166)
- G-protein coupled receptor protein signaling pathway GO:0007186)

References

Adachi, J., C. Kumar, Y. Zhang, J.V. Olsen, and M. Mann. 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biol*. 7:R80.

Ansell, G.B., J.N. Hawthorne and R.M.C. Dawson. 1973. Form and function of phospholipids. Elsevier, Amsterdam. 494 pp.

Antonny, B., S. Beraud-Dufour, P. Chardin, and M. Chabre. 1997. N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry*. 36:4675-84.

Blom, N., S. Gammeltoft, and S. Brunak. 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol*. 294:1351-62.

Brown, R.L., T.L. Haley, K.A. West, and J.W. Crabb. 1999. Pseudechetoxin: a peptide blocker of cyclic nucleotide-gated ion channels. *Proc Natl Acad Sci U S A*. 96:754-9.

Burlak, C., A.R. Whitney, D.J. Mead, T. Hackstadt, and F.R. Deleo. 2006. Maturation of human neutrophil phagosomes includes incorporation of molecular chaperones and endoplasmic reticulum quality control machinery. *Mol Cell Proteomics*. 5:620-34.

Chang, J.T., and J.R. Nevins. 2006. GATHER: a systems approach to interpreting genomic signatures. *Bioinformatics*. 22:2926-33.

Datta, S.A., Z. Zhao, P.K. Clark, S. Tarasov, J.N. Alexandratos, S.J. Campbell, M. Kvaratskhelia, J. Lebowitz, and A. Rein. 2007. Interactions between HIV-1 Gag molecules in solution: an inositol phosphate-mediated switch. *J Mol Biol*. 365:799-811.

Eberle, H.B., R.L. Serrano, J. Fullekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, and J.B. Helms. 2002. Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex. *J Cell Sci*. 115:827-838.

Farazi, T.A., G. Waksman, and J.I. Gordon. 2001. The biology and enzymology of protein N-myristoylation. *J Biol Chem*. 276:39501-4.

Fielding, C.J., and P.E. Fielding. 2003. Relationship between cholesterol trafficking and signaling in rafts and caveolae. *Biochim Biophys Acta*. 1610:219-28.

Gibbs, G.M., M.J. Scanlon, J. Swarbrick, S. Curtis, E. Gallant, A.F. Dulhunty, and M.K. O'Bryan. 2006. The cysteine-rich secretory protein domain of Tpx-1 is related to ion channel toxins and regulates ryanodine receptor Ca2+ signaling. *J Biol Chem*. 281:4156-63.

Gkantiragas, I., B. Brugger, E. Stuven, D. Kaloyanova, X.Y. Li, K. Lohr, F. Lottspeich, F.T. Wieland, and J.B. Helms. 2001. Sphingomyelin-enriched microdomains at the Golgi complex. *Mol Biol Cell*. 12:1819-1833.

Guarino, L.A., G. Smith, and W. Dong. 1995. Ubiquitin is attached to membranes of baculovirus particles by a novel type of phospholipid anchor. *Cell*. 80:301-309.

Ichimura, Y., Y. Imamura, K. Emoto, M. Umeda, T. Noda, and Y. Ohsumi. 2004. In vivo and in vitro reconstitution of Atg8 conjugation essential for autophagy. *J Biol Chem*. 279:40584-92.

Lee, H.K., A.K. Hsu, J. Sajdak, J. Qin, and P. Pavlidis. 2004. Coexpression analysis of human genes across many microarray data sets. *Genome Res*. 14:1085-94.

Li, S., Y. Shin, K.W. Cho, and C.S. Merzdorf. 2006. The Xfeb gene is directly upregulated by Zic1 during early neural development. *Dev Dyn*. 235:2817-27.

Marsh, D. 1990. CRC Handbook of Lipid Bilayers.

McLaughlin, S., and A. Aderem. 1995. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. *Trends Biochem Sci*. 20:272-6.

McLaughlin, S., J. Wang, A. Gambhir, and D. Murray. 2002. PIP(2) and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct*. 31:151-75.

Milano, S.K., Y.M. Kim, F.P. Stefano, J.L. Benovic, and C. Brenner. 2006. Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding. *J Biol Chem*. 281:9812-23.

Milne, T.J., G. Abbenante, J.D. Tyndall, J. Halliday, and R.J. Lewis. 2003. Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. *J Biol Chem*. 278:31105-31110.

Morrissette, J., J. Kratzschmar, B. Haendler, R. el-Hayek, J. Mochca-Morales, B.M. Martin, J.R. Patel, R.L. Moss, W.D. Schleuning, R. Coronado, and et al. 1995. Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. *Biophys J*. 68:2280-8.

Munro, S. 2002. Organelle identity and the targeting of peripheral membrane proteins. *Curr Opin Cell Biol*. 14:506-514.
Obenauer, J.C., L.C. Cantley, and M.B. Yaffe. 2003. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res*. 31:3635-41.
Okamoto, T., A. Schlegel, P.E. Scherer, and M.P. Lisanti. 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem*. 273:5419-22.
Pfeffer, S., and D. Aivazian. 2004. Targeting Rab GTPases to distinct membrane compartments. *Nat Rev Mol Cell Biol*. 5:886-96.
Pisitkun, T., R.F. Shen, and M.A. Knepper. 2004. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 101:13368-73.
Prehoda, K.E., J.A. Scott, R.D. Mullins, and W.A. Lim. 2000. Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science*. 290:801-6.
Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta*. 1451:1-16.
Serrano, R.L., A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, and M.R. Groves. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. *J Mol Biol*. 339:173-83.
Wang, F., H. Li, M.N. Liu, H. Song, H.M. Han, Q.L. Wang, C.C. Yin, Y.C. Zhou, Z. Qi, Y.Y. Shu, Z.J. Lin, and T. Jiang. 2006. Structural and functional analysis of natrin, a venom protein that targets various ion channels. *Biochem Biophys Res Commun*. 351:443-8.
Yamazaki, Y., H. Koike, Y. Sugiyama, K. Motoyoshi, T. Wada, S. Hishinuma, M. Mita, and T. Morita. 2002. Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. *Eur J Biochem*. 269:2708-15.
Yeung, T., B. Ozdamar, P. Paroutis, and S. Grinstein. 2006. Lipid metabolism and dynamics during phagocytosis. *Curr Opin Cell Biol*. 18:429-37.
Zacharias, D.A., J.D. Violin, A.C. Newton, and R.Y. Tsien. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science*. 296:913-6.

Samenvatting in het Nederlands

Inleiding

"Golgi-Associated Plant Pathogenesis-Related protein 1" (GAPR-1) is een eiwit dat voorkomt in zoogdiercellen en dat sterk geconserveerd is onder gewervelde diersoorten. Het komt voornamelijk tot expressie in cellen die een rol spelen in de afweer, zoals monocyten en T-cellen. GAPR-1 komt structureel het sterkst overeen met pathogeen gerelateerde eiwitten groep 1 in planten (plant Pathogen Related proteins, group 1; PR-1). Deze eiwitten spelen waarschijnlijk een belangrijke rol in de afweer tegen pathogenen. De functie van GAPR-1 is tot dusver onbekend, maar de gelijkenis met PR-1 eiwitten en de expressie in immunologisch gerelateerde cellen suggereren dat GAPR-1 is betrokken bij de afweer.

Hoewel GAPR-1 wel sterk lijkt op PR-1 eiwitten, zijn er ook belangrijke verschillen. Aan de N-terminus van GAPR-1 wordt bijvoorbeeld myristinezuur, een vetzuur, gekoppeld. Dit proces heet "myristoylatie", en komt niet voor bij PR-1 eiwitten. Een ander belangrijk verschil tussen GAPR-1 en PR-1 eiwitten is de cellulaire lokalisatie. PR-1 eiwitten worden uitgescheiden na productie door plantencellen. GAPR-1 wordt niet uitgescheiden, maar is stabiel gebonden aan de cytosolaire kant van Golgi complex. Daar bindt het aan zogenaamde "lipid rafts". Dit zijn gespecialiseerde membraandomeinen die een rol kunnen spelen in de doorgifte van cellulaire signalen of het sorteren van eiwitten. Een kleine fractie van de in de cel aanwezige GAPR-1 wordt aangetroffen in het cytosol of op andere organellen dan het Golgi. Zo zit GAPR-1 bijvoorbeeld tijdens fagocytose op fagosomen. Hoe GAPR-1 specifiek naar membranen zoals die van het Golgi en fagosomen gelokaliseerd wordt, is niet bekend.

Het ontrafelen van de membraan-bindingseigenschappen van GAPR-1 zou inzicht kunnen bieden in hoe membraanbinding van GAPR-1 gereguleerd is. Er is een aantal factoren dat bij zou kunnen dragen aan de membraanbinding van GAPR-1. In dit onderzoek hebben we deze factoren onderzocht, voornamelijk met behulp van membraan-bindingsassays. De resultaten die behaald zijn in dit onderzoek suggereren dat binding van GAPR-1 aan membranen afhankelijk is van meerdere factoren die verderop in dit hoofdstuk verder toegelicht zullen worden: 1) binding aan negatief geladen lipiden, 2) binding of koppeling aan fosfatidylinositol, 3) fosforylatie, 4) koppeling aan myristinezuur, 5) dimerisatie (dmv fytinezuur). Hiernaast was uit eerder onderzoek gebleken dat GAPR-1 aan het membraaneiwit caveoline-1 bindt. Al deze factoren maken regulatie van GAPR-1 op verschillende niveaus mogelijk.

1. GAPR-1 bindt aan negatief geladen lipiden

GAPR-1 heeft een isoelectrisch punt van 9.4 en het is daarom positief geladen bij een fysiologische pH, zoals die bij het Golgi. De positieve lading is geconcentreerd in een gebied aan de oppervlakte van GAPR-1, waarmee het waarschijnlijk aan negatief geladen lipiden in het membraan kan binden. Data in **hoofdstuk 2** toonden aan dat niet gemyristoyleerde GAPR-1 inderdaad aan membranen met negatief geladen lipiden kan binden. De mate van binding van GAPR-1 aan membranen correleert met de concentratie van negatief geladen lipiden in die membranen. In **hoofdstuk 2** werd aangetoond dat GAPR-1 aan alle geteste lipiden met slechts één negatieve lading kan binden. GAPR-1 heeft een hogere affiniteit voor fosfatidezuur, wat gedeeltelijk verklaard kan worden door het feit dat dit lipide bij een neutrale pH meer dan één negatieve lading heeft (ca. 1,3). Fosfatidylserine en fosfatidylinositol (PI) zijn de meest voorkomende negatief geladen lipiden in biologische membranen en daarom dragen zij waarschijnlijk het meeste bij aan de elektrostatische interacties van GAPR-1 met membranen. Het is niet uitgesloten dat lokaal hoge concentraties van bijvoorbeeld fosfatidezuur in specifieke delen in het membraan kunnen voorkomen en dat het daardoor een belangrijke factor kan worden in de binding van GAPR-1 met membranen.

In **hoofdstuk 4** hebben we aangetoond dat GAPR-1 een hoge affiniteit heeft voor fosfoinositiden. Fosfoinositiden zijn gefosforyleerde derivaten van fosfatidylinositol en spelen een belangrijke rol in de doorgifte van cellulaire signalen. Er zijn meerdere fosfoinositide varianten, die elk naar een specifiek organeltype lokaliseren. Hierdoor dragen deze lipiden bij aan het bepalen van de identiteit van een organel. Fosfoinositiden zijn op membranen *in vivo* in geringe hoeveelheden aanwezig. Tijdens cellulaire processen waarmee membraan dynamiek gepaard gaat, kunnen echter lokaal hoge concentraties van fosfoinositiden gegenereerd worden. Cytosolaire eiwitten kunnen deze fosfoinositiden binden met behulp van specifieke fosfoinositide bindingsdomeinen en worden hierdoor naar het organel gerekruteerd. Dit zou een manier kunnen zijn waarop GAPR-1 aan specifieke organellen bindt. De hoge affiniteit van GAPR-1 voor fosfoinositiden zou gedeeltelijk verklaard kunnen worden door elektrostatische interacties, aangezien fosfoinositiden sterk negatief geladen zijn en GAPR-1 sterk positief. Echter, de binding van GAPR-1 aan fosfatidylinositol-4,5-bisfosfaat [$PI(4,5)P_2$] is sterker dan die aan fosfatidylinositol-3,4,5-trifosfaat, dat sterker negatief geladen is. Dit impliceert dat GAPR-1 naast lading ook andere kenmerken van lipideklassen herkent. GAPR-1 bindt aan $PI(4,5)P_2$, wat *in vivo* lokaliseert naar het plasmamembraan, terwijl GAPR-1 voornamelijk

gevonden wordt op het Golgi complex. Blijkbaar is de binding aan fosfoinositiden dus niet de enige membraan-lokalisatiefactor, maar het zou wel kunnen bijdragen aan de membraanbinding van GAPR-1.

2. GAPR-1 bindt zeer sterk aan fosfatidylinositol

Tijdens screening voor de binding van GAPR-1 aan negatief geladen lipiden (**hoofdstuk 2**) bleek dat GAPR-1 uitzonderlijk sterk bindt aan PI. De PI binding van GAPR-1 was bestand tegen extractie met organische oplosmiddelen, analyse met behulp van massaspectrometrie en het opkoken onder reducerende omstandigheden. De binding van GAPR-1 aan PI resulteerde in membraanbinding van GAPR-1. Er zijn enkele andere eiwitten die gemodificeerd worden met een fosfolipide. Het eiwit Atg8, wat een rol speelt in autofagocytose, wordt gemodificeerd met fosfatidyl-ethanolamine. Dit leidt tot een conformatieverandering van Atg8 en tot binding van Atg8 membranen. Lipide koppeling zou dus kunnen werken als een moleculaire schakelaar. Een andere fosfolipide koppeling is vermeld voor het eiwit "ubiquitine" tijdens infectie van cellen door baculovirussen. Ubiquitine bindt onder normale omstandigheden niet sterk aan membranen. Echter, door koppeling aan een lipide wordt ubiquitine gelokaliseerd naar het viruspartikel. Dit zou een mechanisme van het virus kunnen zijn om ubiquitine en mogelijk ook andere eiwitten te kunnen wegvangen en te herlokaliseren naar het virale membraan. In de deze voorbeelden leidt lipidebinding tot membraanlokalisatie van een perifeer eiwit. De binding van PI aan GAPR-1 zou dus voor hetzelfde doel kunnen dienen.

3. Myristoylatie draagt bij aan de membraanbinding van GAPR-1

De myristoylatie van GAPR-1 *in vivo* zou de interactie van GAPR-1 met membranen kunnen versterken of stabiliseren. Het gebonden hydrofobe myristinezuur zou namelijk GAPR-1 aan het hydrofobe membraan kunnen verankeren. De bijdrage van de myristoylatie aan de membraanbindings-eigenschappen van GAPR-1 was onderzocht in **hoofdstuk 4**. Een mutatie in de aminozuurvolgorde, die vereist is voor myristoylatie van GAPR-1, resulteerde in de aanmaak van een GAPR-1 eiwit dat niet gemyristoyleerd was. Deze GAPR-1 mutant was gedeeltelijk (30%) te vinden in het cytosol. Echter, bijna alle wildtype GAPR-1 was membraan gebonden. Deze resultaten wijzen erop dat myristoylatie bijdraagt aan membraanbinding van GAPR-1.

4. Membraanbinding van GAPR-1 zou gereguleerd kunnen worden door fosforylatie

Zoals hierboven beschreven is, heeft GAPR-1 verscheidende karakteristieken die membraanbinding bevorderen: een positief geladen deel op de eiwitoppervlakte, een interactie met een membraangebonden eiwit (caveoline-1), een myristoylatie en de binding aan fosfatidylinositol. Volgens het "two-signal" membraan-bindingsmodel van M. Resh zijn twee van zulke eigenschappen voldoende voor een stabiele membraanbinding. GAPR-1 is in cellen sterk membraangebonden. Echter, een kleine hoeveelheid GAPR-1 lokaliseert naar het cytosol, wat aangeeft dat onder bepaalde omstandigheden GAPR-1 van membranen losgemaakt kan worden. In **hoofdstuk 4** is door middel van een metabole labeling met radioactief fosfaat aangetoond dat de cytosolisch gelokaliseerde GAPR-1 sterk gefosforyleerd is in vergelijking tot membraangebonden GAPR-1. Door fosforylatie wordt een negatieve lading toegevoegd aan GAPR-1, wat de affiniteit van GAPR-1 voor negatief geladen membraanlipiden zou kunnen verminderen. Vermindering van de affiniteit voor membranen door fosforylatie is al eens beschreven voor MARCKS eiwitten. Het verbreken van de elektrostatische interacties met het membraan door fosforylatie resulteert in de verplaatsing van MARCKS eiwitten vanaf membranen naar het cytosol. Dit mechanisme staat bekend als de "myristoyl-electrostatic switch". Het is mogelijk dat voor regulatie van membraanbinding van GAPR-1 ook gebruik gemaakt wordt van een dergelijk mechanisme.

Loslating van GAPR-1 vanaf het membraan naar het cytosol vereist afscherming van het gebonden hydrofobe myristinezuur voor een waterige omgeving. Andere eiwitten met hydrofobe modificaties hebben verscheidende strategieën om deze modificaties af te schermen. Rab eiwitten binden aan membranen met een of twee hydrofobe prenylgroepen. Bij inactivatie van Rab eiwitten kunnen "GDP dissociation inhibitor" (GDI) eiwitten de prenylgroepen afschermen van het cytosol, waardoor Rab eiwitten het membraan kunnen loslaten. Fosforylatie zou ook binding van GAPR-1 aan cytosolaire eiwitten mogelijk kunnen maken, die competeren met factoren die betrokken zijn in membraanbinding. Dit zou kunnen resulteren in het loslaten van GAPR-1 van het membraan. Een andere mogelijkheid is dat fosforylatie leidt tot een conformatieverandering van GAPR-1, dat loslating van het membraan mogelijk maakt. Voor het eiwit Arf1 was aangetoond dat het een conformatieverandering kan ondergaan, waardoor het de myristinezuurgroep kan afschermen in een hydrofobe uitsparing op het eiwitoppervlak. Dit mechanisme staat bekend als een "myristoyl-conformational switch". Het moet nog onderzocht worden of gefosforyleerd GAPR-1

ook loslaat via een dergelijk mechanisme, aangezien nog geen duidelijke hydrofobe uitsparing is gevonden in de GAPR-1 structuur.

5. Dimerisatie van GAPR-1 door fytinezuur

De sterke binding van GAPR-1 aan PI en fosfoinositiden suggereert dat GAPR-1 goed bindt aan inositol of inositolfosfaten, wat de kopgroepen zijn van deze lipiden (zie Fig 1, hoofdstuk 1 voor de struktuur van lipiden en Fig 2A, hoofdstuk 1 voor de struktuur van PI). Een dergelijke binding zou mogelijk membraanbinding van GAPR-1 kunnen beïnvloeden. Daarom hebben we in **hoofdstuk 3** onderzocht of GAPR-1 kan binden aan inositolfosfaten. Hierbij hebben we de binding van GAPR-1 aan het inositolderivaat fytinezuur onderzocht. Fytinezuur is zes keer gefosforyleerd. Het komt in hoge concentraties in planten voor, maar het is ook aanwezig in zoogdiercellen. De binding van GAPR-1 aan fytinezuur bleek sterk te zijn. Het induceert dimerisatie van GAPR-1 en het remt de membraanbinding van GAPR-1. Dimerisatie van GAPR-1 op zichzelf remt de membraanbinding niet, omdat een mutant van GAPR-1, welke een sterke neiging heeft om dimeren te vormen, verhoogde membraanbinding liet zien *in vitro*. De vondst van GAPR-1 dimeren op Golgi membranen *in vivo* versterkt onze conclusie dat dimeerformatie niet remmend werkt op membraanbinding. Dit wijst erop dat fytinezuur op een andere manier de membraanbinding van GAPR-1 reguleert. Om te onderzoeken wat voor een effect fytinezuurbinding op GAPR-1 heeft op moleculair niveau, werd GAPR-1 samen met fytinezuur gekristalliseerd. Vervolgens werd aan de hand van dit kristal de moleculaire structuur van het fytinezuur-GAPR-1 complex opgehelderd, welke vergeleken kon worden met die van de al bekende ongebonden GAPR-1. Hieruit bleek dat fytinezuur-gebonden GAPR-1, net zoals ongebonden GAPR-1, gedimeriseerd is. Echter, fytinezuur-gebonden GAPR-1 had een andere conformatie dan ongebonden GAPR-1. In vergelijking tot ongebonden GAPR-1 waren de GAPR-1 monomeren in de dimeer van fytinezuur-gebonden GAPR-1 ten opzichte van elkaar gedraaid. Deze rotatie zou verantwoordelijk kunnen zijn voor de geobserveerde remming van membraanbinding van GAPR-1 in de aanwezigheid van fytinezuur. Dit vermoeden werd bevestigd doordat membraanbinding van een mutant, die deze draaiing niet kon maken, ongevoelig bleek te zijn voor fytinezuurbinding. De cellulaire concentratie van fytinezuur ligt dicht bij de concentratie waarbij binding van GAPR-1 aan membranen *in vitro* gehalveerd wordt. Daarom zou fytinezuur membraanbinding van GAPR-1 *in vivo* kunnen reguleren. Het is ook mogelijk dat niet het fytinezuur zelf, maar dat structureel gerelateerde moleculen de membraanbinding van GAPR-1

reguleren. Op basis van de gelijkenis van de kopgroepen van de fosfoinositiden met fytinezuur is het aanlokkelijk om te speculeren dat deze lipiden niet alleen betrokken zijn in de membraanbinding van GAPR-1, maar ook in de regulatie van GAPR-1 dynamiek. GAPR-1 lijkt alle fosfoinositiden te binden (**hoofdstuk 4**), maar er is wel wat specificiteit geobserveerd aangezien bijvoorbeeld GAPR-1 minder binding aan $PI(3,4,5)P_3$ liet zien dan aan $PI(4,5P)_2$. Dus de dynamiek en binding van GAPR-1 zou beïnvloed kunnen worden door een verandering van de fosfoinositidesamenstelling in membranen. Dit suggereert een regulatie via een fosfaat gemedieerde schakelaar, waarmee cellen dmv inositolfosfaten de locatie van GAPR-1 kunnen bepalen.

Conclusie

De resultaten in dit proefschrift laten duidelijk zien dat membraanbinding van GAPR-1 afhankelijk is van meerdere factoren.

Factoren die membraanbinding van GAPR-1 stimuleren zijn:

1) Aanwezigheid van negatief geladen lipiden, in het bijzonder fosfoinositiden, in het membraan
2) de myristoylatie van GAPR-1
3) sterke binding van GAPR-1 aan fosfatidylinositol
4) binding van GAPR-1 aan membraaneiwitten.

Factoren die een lokalisatie van GAPR-1 naar het cytosol bevorderen zijn:

1) Fosforylatie van GAPR-1
2) Rotatie van de GAPR-1 monomeren in een dimeer dmv binding aan inositolfosfaten
3) binding aan cytosolaire eiwitten.

De grote hoeveelheid factoren geeft de cel meerdere mogelijkheden om de lokalisatie van GAPR-1 te reguleren. De resultaten zouden kunnen helpen om inzicht te krijgen hoe de dynamiek van GAPR-1 of van andere perifere membraaneiwitten gereguleerd zouden kunnen zijn.

Dankwoord

OK dan, het is zover. Ik kan nu bijna zeggen dat het af is. Nog niet helemaal, want ik wil nog de mensen bedanken die me geholpen of gesteund hebben tijdens deze hele periode. Ik heb met zeer veel plezier op deze afdeling gewerkt, dus daar wil ik al mijn collega´s sowieso voor bedanken. Toch wil ik ook een aantal mensen specifiek bedanken die me erg hebben geholpen.

Bernd, ik wil je enorm bedanken voor je hulp als promotor en begeleider. Ik heb die jaren met veel plezier met je samengewerkt. Ik heb het altijd gewaardeerd dat je, ondanks je uitpuilende agenda, toch meestal tijd had om resultaten, plannen of manuscripten met mij te bespreken. Deze besprekingen heb ik altijd erg leuk en motiverend gevonden, vooral vanwege je constructieve en analytische benadering van zaken. Ook wil ik je bedanken voor het mogelijk maken van het komende buitenlandse avontuur.

Mijn co-promotoren zou ik graag willen bedanken voor hun hulp en ideeën. Dora, bedankt voor jouw creativiteit en adviezen tijdens de dropjes/koekjes-brainstorm-sessies. Het was altijd een goed idee om je kamer binnen te vallen om van alles en nog wat door te nemen en dingen in een ander perspectief te plaatsen. Coos, bedankt voor je hulp bij het moleculaire werk, vooral in de eerste jaren. Ook bedankt voor het zeer nauwkeurig nakijken van de manuscripten.

Mijn directe labgenoot Ruud, ontzettend bedankt voor alle hulp. Het is teveel om het allemaal nu op te noemen, maar je stond in ieder geval altijd voor me klaar. Fijn ook dat je mijn paranimf wilde zijn. Ik vond het erg leuk om met je samengewerkt te hebben, want het was altijd gezellig met jou op het lab en daarbuiten.

Ook zou ik bij deze de mensen willen bedanken die direct met me hebben samengewerkt en die me hebben geholpen met de experimenten. Piet en Arie, bedankt voor de leuke samenwerking. Ik vond het altijd leuk om langs te komen, vooral omdat het meestal vele creatieve ideeën en interessante theorieën opleverde. Bas v. B., bedankt voor de MS analyse. Het was een hele uitdaging, want het was zeker geen alledaags sample. Ik ben dus blij dat je er zo enthousiast op gedoken bent. Peter and Leo, thank you for providing the huge amount of recombinant GAPR-1 and also for the setup of the liposome binding assay, it has been proven to be very useful. Jos en Lilian, bedankt voor jullie hulp met de MS analyse. Ook ben ik erg blij met de hulp voor het komende project. Ik hoop dat daar mooie resultaten uit voort zullen komen. Chris, bedankt voor al die keren dat je me hebt geholpen met

allerhande kolommen, vooral de FPLC. Wil en Martin H., bedankt voor jullie hulp met de TLC en andere proeven. Marco en Raymond, bedankt voor jullie hulp met de celkweek. En Raymond, je kaarten waren erg handig, ik gebruik ze nog steeds! Danny, bedankt voor het helpen met kloneren.

Ook mijn kamergenoten zou ik graag willen bedanken voor de goede sfeer. Ik kon altijd iets vragen, klagen of opgepept worden. Esther B., je invoering van het power uur en je dreiging om tabellen te moeten maken als je je er niet aan hield, hebben me erg goed geholpen. Ook ben ik blij dat we samen hebben kunnen vaststellen wat nu precies ROR is en wat niet. Kim, fijn dat ik bij je mocht "afkijken", vooral bij de regeldingen aan het eind. Zo hoefde ik het wiel niet nog eens uit te vinden. Marion, wat betreft ARBO vragen zat ik altijd goed bij jou. Ook ben ik blij dat je me met de FPLC hebt kunnen helpen. Rachid, we hebben niet lang de kamer gedeeld, maar het was gezellig voor the time being.

Koen, Ynske, Mijke, Leonie, Reinard en Mokrish, jullie waren erg leuke buren. Het was dan ook geen toeval dat ik meestal via jullie kamer naar de mijne liep. Een tussenstop in jullie kamer leverde meestal een leuk praatje, een koekje of snoep op. Bedankt daarvoor!

Willem, Richard, Lodewijk, Jaap, Bas V., Bart, Martin v E., de discussies met jullie, bijvoorbeeld aan de koffietafel, heb ik altijd erg leuk gevonden.

Judith, bedankt voor je vrolijke aanwezigheid en het snelle regelen van allerlei zaken. Een verlopen pasje meestal in een oogwenk weer geactiveerd.

Van de afdeling Multimedia zou ik graag Harry Otter willen bedanken voor de layout en Marije Brouwer voor het ontwerp van de kaft. Het eindresultaat kan ik natuurlijk nog niet zien, maar het ziet er al veelbelovend uit!

Arjan, fijn dat je me wilde opvolgen in de IB AIO commissie.

Nicole, Esther V., Ruud, Marian en Tjeerd, bedankt voor de geboden weerstand bij Puerto Rico, Carcassonne en Kolonisten van Catan en natuurlijk ook voor het bijbehorende eten.

Jan-Willem, Evert, Karin, Sonja, Toine, Onno, Martin v. E., Jason, Gerrianne, Johanna, Rachid A., Núria, bedankt voor de gezelligheid in en buiten het lab.

Ook de rest van mijn collega´s op de afdeling wil ik bedanken voor de gezelligheid en behulpzaamheid.

Ook van buiten het lab heb ik de nodige hulp gehad: Silvester, fijn dat je paranimf wilde zijn. Hans, dank je voor de adviezen voor de layout. Zoals je ziet is het een en ander wel ingevoerd. Lude, Pieter en Koen, de "spelletjesavonden" waren

vrij apart in het opzicht dat er geen spelletjes werden gespeeld. In plaats daarvan werd er gekletst, maar er werd ook wetenschap besproken. Dit heeft leuke ideeën opgeleverd. Ook de andere mede-Mebioten, maar ook de D&Ders, MTGers, huisgenoten en de rest van mijn omgeving wil ik bedanken voor hun support.

Lauke, Veerle, Kjell, Marije en Joost, bedankt voor jullie belangstelling tijdens de hele periode.

Hetty en Peter, lieve ouders, ik ben jullie ontzettend dankbaar voor de enorme steun die jullie, samen met Jan en Mieke, me de afgelopen jaren hebben gegeven. Jullie interesse en vertrouwen, dat het uiteindelijk allemaal goed zou komen, hebben zeker positief gewerkt. Bedankt daarvoor!

Bedankt allemaal!

Josse

Curriculum Vitae

Josse van Galen werd op 31 augustus 1979 geboren te Utrecht. In 1997 behaalde hij zijn VWO diploma aan het Spectrum College te Utrecht. In datzelfde jaar begon hij aan de studie Biomedische Wetenschappen aan de Universiteit Utrecht. Tijdens deze studie liep hij zijn bijvakstage bij de vakgroep Dermatologie en Allergologie in het UMC Utrecht onder begeleiding van dr. E. van Hoffen en dr. E.F. Knol. Vervolgens liep hij zijn hoofdvakstage bij de vakgroep Celbiologie in het UMC Utrecht onder begeleiding van dr. R. Wubbolts en dr. W. Stoorvogel. Bij diezelfde groep schreef hij ook zijn afstudeerscriptie. In 2003 behaalde hij zijn doctorale diploma. In hetzelfde jaar begon hij onder begeleiding van prof. dr. J.B. Helms aan een promotieonderzoek op de afdeling Biochemie en Celbiologie aan de Faculteit Diergeneeskunde van de Universiteit Utrecht. Dit onderzoek heeft tot dit proefschrift geleid.

Vanaf mei 2008 zal hij als postdoc werkzaam zijn in de onderzoeksgroep van prof. dr. V. Malhotra wat deel uitmaakt van het "Center for Genomic Regulation" in het "Barcelona Biomedical Research Park" te Barcelona, Spanje.