# Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression

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The reggie protein family consists of two proteins, reggie-1 and -2, also called flotillins, which are highly ubiquitous and evolutionarily conserved. Both reggies have been shown to be associated with membrane rafts and are involved in various cellular processes such as T-cell activation, phagocytosis and insulin signalling. However, the exact molecular function of these proteins remains to be determined. In addition, the mechanism of membrane association of reggie-1, which does not contain any transmembrane domain, is not known. In this study, we have produced a fusion protein of reggie-1 with enhanced green fluorescent protein and generated targeted substitutions for the inactivation of putative palmitoylation and myristoylation sites. We were able to show that reggie-1 is myristoylated and multiply palmitoylated and that lipid modifications are necessary for membrane association of reggie-1. Overexpression of reggie-1 resulted in the induction of numerous filopodia-like protrusions in various cell lines, suggesting a role for reggie-1 as a signalling protein in actin-dependent processes.

Key words: actin cytoskeleton, filopodia, flotillin, protein acylation, rafts, reggie.

## INTRODUCTION

The reggie proteins, also named flotillins, were originally described as axonal regeneration proteins up-regulated after a lesion of the goldfish optic nerve [1]. The two reggies are homologous with each other and show a very profound evolutionary conservation [2], suggestive of an important but as yet unknown cellular function. An important function for reggies is also suggested by the ubiquitous expression of especially reggie-1 which is present in virtually all cell lines we have tested to date and in most tissues ([3] and our unpublished work).

Reggies have been shown to be associated with detergent-insoluble membrane rafts and to co-localize with clustered GPI (glycosylphosphatidylinositol)-anchored proteins in rafts in neurons and T-lymphocytes [4,5]. Consistently, immunostaining of these cells reveals a patchy labelling pattern at the plasma membrane, especially in the growth cones of axons and filopodia, where the two reggies partially co-localize. In addition, they co-localize with Fyn kinase and activated GPI-linked cell adhesion molecules such as F3 and Thy-1 in rafts in neurons and lymphocytes [5]. The rafts containing reggies have been suggested to be identical with caveolae [6]. However, other studies have shown that reggies are raft-associated also in cells that do not contain any caveolae and that they rather localize in a non-caveolar raft also in non-neuronal cells ([4,5,7] and our unpublished work). Recent findings in adipocytes showed that flotillin-1/reggie-2 does not co-purify with caveolae and thus resides in another raft type [8]. The association of reggies with signalling molecules in rafts would suggest a role in signal transduction. In agreement with that, recent studies have revealed a role for the reggie proteins in various cellular processes such as endocytosis, phagocytosis and insulin signalling [5,9,10,12].

Previous studies have suggested that reggie-2/flotillin-1 would be a single-spanning transmembrane protein containing a short extracellular segment and a larger cytoplasmic domain [6,11]. However, recent findings from Morrow et al. [13] showed that reggie-2 is protected from protease digestion in non-permeabilized cells and is thus probably cytoplasmic, at least for a large part. This study also showed that reggie-2 associates with membranes by means of a single palmitate modification in a conserved Cys-34. In line with its similarity to reggie-2, reggie-1/flotillin-2 does not seem to contain any typical transmembrane domain, and the exact mode of membrane association of reggie-1 is not known. It is thus unclear if it is totally cytoplasmic or if part of the protein reaches the cell surface through the plasma membrane. Immunostaining of cells with reggie-specific antibodies requires permeabilization of the membranes, suggesting an intracellular location, identical with reggie-2.

Several studies have shown that lipid modifications (myristoylation and palmitoylation) of proteins play a role in the association with raft membranes. Myristoylation is a co-translational, irreversible process that can take place in a glycine residue immediately after the translation-initiation codon. Palmitoylation (thioacylation) is a reversible modification of cysteine residues, which takes place post-translationally in a consensus sequence Gly-Xaa-Cys and might function in the regulation of the localization of proteins in cell membranes. If a protein is both myristoylated and palmitoylated, myristoylation is a pre-requisite for palmitoylation to take place [14]. Many signalling proteins that reside on the inner side of the plasma membrane, such as the non-receptor tyrosine

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; FCS, foetal calf serum; FL, full-length; FRT, Fischer rat thyroid; SH, short; SPFH, stomatin, prohibitin, flotillin homology; WT, wild-type; X-$\alpha$-Gal, 5-bromo-4-chloroindol-3-yl $\alpha$-D-galactopyranoside; YFP, yellow fluorescent protein.

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kinases Lck and Fyn, are both myristoylated and palmitoylated. These modifications have also been shown to mediate their raft association in a regulated fashion. In addition, oligomerization, polybasic protein domains and prenylation have been indicated in the regulation of protein distribution in subcellular structures and membrane domains [15,16]. Although raft-associated proteins are frequently myristoylated and palmitoylated, prenylation is rarely observed [17].

The aim of the present study was to shed light on the mechanisms of membrane and raft association of reggie-1, the more ubiquitous one of the two reggies. Fusion proteins of reggie-1 with enhanced green fluorescent protein (EGFP) were used to study the putative lipid modifications and their role in the association of reggie-1 with membranes. In addition, oligomerization of reggie-1 as a putative determinant for raft association was studied. Overexpression of reggie-1 was found to induce filopodia in several epithelial cells, indicating a role for reggie-1 in the reorganization of actin cytoskeleton.

## EXPERIMENTAL

### Plasmid cloning

Rat reggie-1 cDNA (GenBank® accession no. AF 023302) cloned in the plasmid pBluescript [4] was used as a PCR template to create various reggie-1-EGFP fusion constructs. An FL (full-length) variant and a truncated variant containing amino acids 1–30 were generated. In addition to a wild-type construct, single-point mutations Gly2Ala, Cys4Ala, and double/triple mutants Cys4 + 19Ala and Cys4 + 19 + 20Ala were produced in both variants. The mutations were inserted by PCR with specific primers, and the products were cloned into the vector pEGFP-N1 (ClonTech Laboratories, Palo Alto, CA, U.S.A.), resulting in the C-terminal fusion of the EGFP tag with reggie-1. As a control, the plasmid pEYFP-mem (where YFP stands for yellow fluorescent protein; ClonTech Laboratories) encoding a plasma-membrane-associated YFP fusion protein was used.

### Cell culture and transfection

FRT (Fischer rat thyroid) cells were cultured in F-12 (Coon's) medium containing 5 % FCS (foetal calf serum). HaCaT cells (a human keratinocyte cell line, [18]) and HeLa cells (human cervix adenocarcinoma cells) were maintained in DMEM (Dulbecco's modified Eagle's medium; Cambrex, East Rutherford, NJ, U.S.A.) containing 10 % FCS.

For fluorescence microscopy, the cells were transfected using the transfection reagent Escort IV (Sigma) as recommended by the manufacturer. HeLa cells were used for all biochemical studies as they ensure the highest transfection rates. For biochemical studies, HeLa cells were transfected using the Metafectene reagent (Biontex, Munich, Germany) as recommended by the manufacturer, and lysed or prepared for the experiments 24–48 h post-transfection.

### Inducible expression of reggie-1-EGFP

The plasmid pInd was obtained from Invitrogen (Groningen, Netherlands). A 2 kb fragment encoding a fusion of reggie-1 with EGFP was isolated as an *Eco*RI–*Not*I fragment from reggie-1-EGFP1-EGFP and was ligated into the *Eco*RI–*Not*I-digested pInd. In the resulting plasmid pER-reggie-1-EGFP, reggie-1-EGFP expression was under the control of an ecdysone-responsive promoter.

The plasmid pER-reggie1-EGFP was transfected into BGL109 cells [19] and cell clones with stable integration of the plasmid were isolated as described previously. One cell clone (BGL236) with inducible transgene expression, as judged by the intensity of GFP fluorescence, was used in this study. The generation of the BGL100 control cells has been described previously [19]. BGL100 cells expressed EGFP in a Muristerone A-concentration-dependent fashion. Cells were maintained in Ham's F12 medium with 10 % FCS and antibiotics, and supplemented with 5 $\mu$M Muristerone A for induction of a transgene expression.

### Sucrose gradient ultracentrifugation

Cells were washed with PBS (150 mM NaCl/20 mM sodium phosphate, pH 7.4), scraped into a lysis buffer (25 mM Mes/150 mM NaCl/60 mM *N*-octylglucoside, pH 6.5) and incubated for 30 min at 4 °C. Lysates were poured on linear sucrose density gradients (5–40 % sucrose) in 5 ml Beckman Ultra-Clear centrifuge tubes. After centrifugation (300 000 ***g***, > 10 h, 4 °C; Beckman SW50.1 rotor), 500 $\mu$l fractions were collected from the top and prepared for SDS/PAGE. This method was standardized using the Sigma molecular-mass marker kit MW-GF-1000.

### Preparation of soluble and membrane-associated protein fractions

Cells were washed with PBS, scraped from the dishes and then homogenized mechanically in a hypotonic buffer (0.25 M sucrose/1 mM EGTA/10 mM Hepes/NaOH, pH 7.2). Nuclei and non-lysed cells were removed by centrifugation for 5 min at 2000 ***g***. The supernatant was collected and the pellet was re-homogenized in the buffer, centrifuged, and the two supernatants were combined. Membrane fragments were pelleted by ultracentrifugation (100 000 ***g***, 4 °C, 1 h; Beckman TLA-55 rotor). The supernatant containing soluble proteins was collected and the remaining membrane pellets were resuspended in lysis buffer (10 mM Tris/HCl/1 mM EDTA/1 mM dithiothreitol/60 mM *N*-octylglucoside/0.5 % Triton X-100, pH 8.0). Both soluble and membrane-associated fractions were analysed by SDS/PAGE and Western blotting. For the detection of the fusion proteins, a monoclonal mouse anti-EGFP (Roche, Mannheim, Germany) or a monoclonal mouse anti-reggie-1/epidermal surface antigen (Transduction Laboratories, BD Pharmingen, Heidelberg, Germany) was used.

### Isolation of detergent-insoluble membrane fractions

Rafts were isolated using the method described by Harder et al. [20] with modifications as in [21]. Transfected cells were washed on ice with PBS and extracted with cold 1 % Triton X-100 in TNE buffer (25 mM Tris/HCl/150 mM NaCl/5 mM EDTA, pH 7.5) for 30 min on ice. The extract was adjusted to 35 % Optiprep and loaded on the bottom of an Optiprep step gradient (35, 30, 0 %). After ultracentrifugation (175 000 ***g***, 4 °C, 4 h; Beckman SW50.1 rotor), 600 $\mu$l of fractions were taken from the top. SDS (2 %) was added to the fractions, which were then prepared for SDS/PAGE.

### Metabolic labelling

HeLa cells were transfected with the reggie-1-EGFP fusion constructs in 6-well plates. For labelling with [$^{35}$S]Cys/Met, the cells were starved for 1 h in a medium without cysteine and methionine and thereafter labelled with 50 $\mu$Ci of [$^{35}$S]Cys/Met for 4 h (ProMix, Amersham Biosciences, Uppsala, Sweden; specific radioactivity, > 1000 Ci/mmol) in DMEM without FCS. To study the fatty acid modifications, the transfected cells were labelled with 100 $\mu$Ci of [$^{3}$H]myristate (54 Ci/mmol) in 0.5 ml

of DMEM without FCS for 4 h or with 200 $\mu$Ci of [$^3$H]palmitate (53 Ci/mmol) in 1 ml of DMEM with 2 % dialysed FCS for approx. 18 h. Thereafter the cells were harvested and prepared for immunoprecipitation.

### Immunoprecipitation

IP buffer (10 mM Tris/150 mM NaCl/5 mM EDTA/0.5 % Triton X-100/60 mM *N*-octylglucoside/10 % BSA) was added to the lysates, which were thereafter precleared by rolling with 50 $\mu$l of Pansorbin beads (1 h, 4 °C). The lysates were incubated (> 10 h, 4 °C) by rolling with 30 $\mu$l of Protein A-Dynabeads (Dynal Biotech, Oslo, Norway), which had been coupled with 2 $\mu$l of rabbit anti-GFP antibody (Becton Dickinson, Franklin Lakes, NJ, U.S.A.; ClonTech Laboratories). The beads were washed with 1 ml each of Neufeld buffer (10 mM Tris/600 mM NaCl/0.1 % SDS/0.05 % Nonidet P40, pH 8.5), IMM buffer (0.5 % sodium deoxycholate in PBS/1 % Triton X-100), IMM buffer with 2 M KCl and 0.1 × PBS. The precipitated proteins were solubilized into SDS/PAGE loading buffer by boiling for 5 min (palmitate labelling at 80 °C for 3 min), and analysed by SDS/PAGE. For metabolic labelling with [$^{35}$S]Cys/Met, the gel was dried and then exposed to an X-ray film. For tritium labels, the gel was blotted on to nitrocellulose and thereafter exposed using Biomax MS X-ray films with an enhancing screen (Kodak). For verification of the specificity of the [$^3$H]palmitate signals, the samples were run on an SDS/polyacrylamide gel, which was then incubated in 1 M hydroxylamine (pH 7.5) with NaOH for 1 h at room temperature (22 °C). This procedure removes the labelling which is associated with thioacyl-linked palmitates in cysteine residues. Afterwards, the gel was immersed for 20 min in blotting buffer [192 mM glycine/25 mM Tris/20 % (v/v) methanol] and then blotted on to nitrocellulose and exposed on a screen.

### Fluorescence microscopy

For fluorescence microscopy of GFPs, the cells were grown on coverslips and transfected as described above. After 24–48 h, the cells were washed with PBS, fixed with methanol (10 min, − 20 °C) and mounted in Gel/Mount (Biomeda, Foster City, CA, U.S.A.) with 50 mg/ml 1,4-diazadicyclo(2,2,2)octane (Fluka, Taufkirchen, Germany). The analysis was performed using a confocal laser scanning microscope (Zeiss LSM 510).

### Yeast two-hybrid analysis

The FL coding region of reggie-1 or fragments thereof were cloned into the yeast two-hybrid vectors as fusion proteins with the DNA binding (pGBKT7) or activation domain (pGADT7) of Gal4 using the Matchmaker3 yeast two-hybrid system (ClonTech Laboratories). In addition to the FL protein, an N-terminal fragment (amino acids 1–233) and a C-terminal fragment (200–428) were produced. All fragments were cloned into both vectors and transformed pairwise into the yeast strain AH109 as instructed by the manufacturer. Positive clones were selected on the basis of growth on nutrient-deficient plates and presence of galactosidase activity according to standard methods.

## RESULTS

To characterize the mechanisms of membrane and raft association of reggie-1 and especially the role of the possible lipid modifications therein, fusion proteins between EGFP and rat reggie-1 were generated. Reggie-1 was C-terminally tagged with EGFP, because the second residue in reggie-1, a glycine, is predicted to become myristoylated. Owing to this, an N-terminal EGFP tag would probably prevent the myristoylation of reggie-1 and consequently also its membrane association. Failure in myristoylation would also result in a lack of further modifications such as palmitoylation, which could occur in Cys-4, Cys-19 or Cys-20 of rat reggie-1.

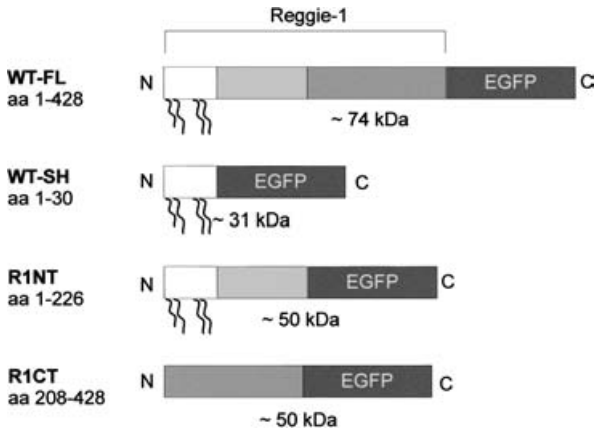


**Figure 1 Reggie-1-EGFP fusion protein constructs**

The complete rat reggie-1 coding region was PCR-amplified and fused in the N-terminus of EGFP. In addition, a truncated construct coding only for the first 30 amino acids of reggie-1 was produced to study directly the effect of the lipid modifications on the raft association of reggie-1. Constructs encoding for either the N-terminal (R1NT) or the C-terminal half (R1CT) of reggie-1 were also produced.

The FL rat reggie-1 coding region was fused to the N-terminus of EGFP (Figure 1). Truncated variants of reggie-1 were also produced in which only the first 30 amino acids were fused with EGFP. The fragment contained all the putative lipid modification sites of reggie-1. In addition to the WT (wild-type) reggie-1, targeted amino acid substitutions for the inactivation of the putative fatty-acid modification signals were produced. A Gly2Ala exchange abolishes the predicted myristoylation signal, whereas Cys4Ala, Cys19Ala and Cys20Ala each disrupt a putative palmitoylation site. All mutations were produced in the FL and SH (short) reggie-1-EGFP constructs. Furthermore, two constructs encoding the N-terminal (amino acids 1–226) or the C-terminal (208–428) half of reggie-1 were produced and the resulting fusion proteins were expressed in HeLa cells. The rate of overexpression as compared with the endogenous reggie-1 was approx. 1:1.

### Reggie-1 is myristoylated and palmitoylated

For the direct demonstration of the fatty-acid modifications, immunoprecipitation after metabolic labelling was used. HeLa cells were transfected with the FL constructs, labelled with tritiated myristate or palmitate, and the fusion proteins were immunoprecipitated with polyclonal antibodies against EGFP. The precipitated proteins were separated by SDS/PAGE, blotted on to a nitrocellulose membrane for enhanced detection of the tritium label, and the blots were exposed for 2–7 days. All other variants except for the Gly2Ala were found to be myristoylated, as shown in Figure 2(A). In the non-transfected control cells (Figure 2A, lane C), no signal could be detected either, demonstrating the specificity of the immunoprecipitation reaction.

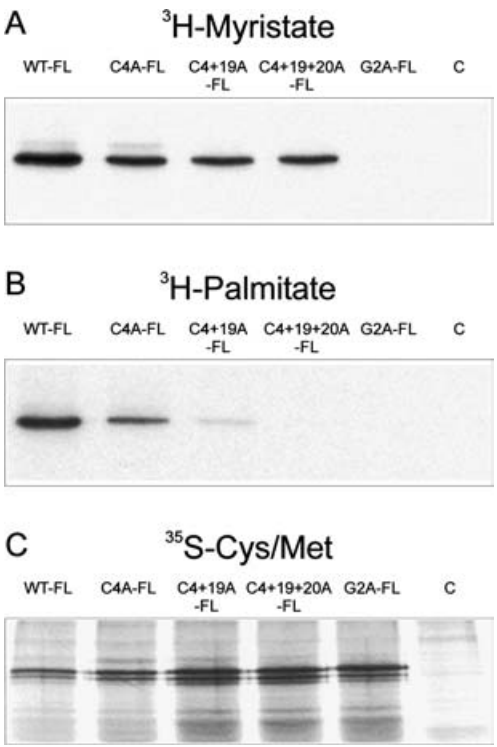


**Figure 2 Myristoylation and palmitoylation of reggie-1**

Immunoprecipitation of reggie-1-EGFP fusion proteins with GFP antibody after metabolic labelling of transfected HeLa cells. (**A**) Labelling with tritiated myristic acid showed that the WT-FL as well as all Cys-Ala substitution mutants were myristoylated, whereas the Gly2Ala-FL was not. No signal could be detected in the control (lane C) either. (**B**) The WT-FL fusion protein was palmitoylated, as revealed by labelling with tritiated palmitic acid. Cys4Ala-FL (C4A-FL) and Cys4 + 19Ala-FL (C4 + 19A-FL) mutants showed successively reduced amounts of palmitoylation, the C4 + 19 + 20A-FL mutant being completely non-palmitoylated. Gly2Ala-FL (G2A-FL) mutant exhibited no palmitoylation, indicating that lack of myristoylation also prevents palmitoylation. (**C**) All fusion protein variants were equally expressed and precipitated by the antibody, as shown by metabolic labelling with [$^{35}$S]Cys/Met. A double band was observed in all transfected samples, probably representing a post-translationally modified or processed form of reggie-1.

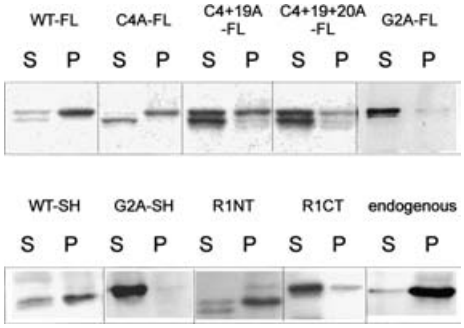


**Figure 3 Membrane association of reggie-1: dependence on the lipid modifications and further protein determinants**

Soluble and membrane-associated protein fractions were prepared from transfected HeLa cells using high-speed centrifugation. FL fusion proteins were detected using a reggie-1-specific antibody, and truncated fusion proteins using a GFP-antibody. S, soluble; P, pellet. Endogenous reggie-1 was found to be almost exclusively membrane-associated, as was the WT-FL fusion protein. C4A-FL fusion protein was detected mostly in the membrane fraction, although it was clearly more soluble than the WT-FL protein. Note the difference in the gel mobility of the soluble and membrane-associated fragments. Destruction of further palmitoylation sites makes the proteins more soluble and results in the appearance of the upper band in the soluble fraction. C4 + 19 + 20A-FL and G2A-FL mutants were found to a large degree in the soluble fraction. The WT-SH fusion protein carrying all lipid modifications was more soluble than WT-FL, indicating that lipid modifications alone are not sufficient for full membrane association, whereas the G2A-SH mutant was soluble. The N-terminal half of reggie-1 (R1NT) was largely membrane-associated, whereas the C-terminal half (R1CT) was virtually soluble.

Metabolic labelling with palmitic acid resulted in an abundant signal in the WT-transfected cells (Figure 2B). A clearly reduced signal (26 % of WT) could be detected for the Cys4Ala variant, indicating that Cys-4 serves as the major palmitoylation site, but other cysteine residues are also palmitoylated. A very low amount of palmitoylation was observed for the mutant Cys4 + 19Ala, whereas no palmitoylation was detected with the triple mutant Cys4 + 19 + 20Ala, indicating that Cys-19 and Cys-20, in addition to Cys-4, can be palmitoylated, although Cys-4 probably represents the major palmitoylation site. The Gly2Ala mutant was not palmitoylated either, consistent with the failure for palmitoylation to occur in the absence of myristoylation. Lack of signal in the mutants and sensitivity to hydroxylamine (results not shown) also indicate that the observed labelling is not due to conversion of the palmitate label into other metabolic products that might be incorporated into the protein.

Immunoprecipitation after metabolic labelling with [$^{35}$S]Cys/Met was used to verify that the absence of signal for the mutants was not due to non-expression or instability of the mutant fusion proteins. As shown in Figure 2(C), all variants were expressed in similar amounts. Pulse–chase experiments with chase times up to 6 h were used to demonstrate that the stability of the mutant proteins was not altered. All fusion proteins showed only minimal degradation after 6 h of chase, demonstrating that the absence of signal in the palmitate/myristate labellings was not due to increased degradation of the mutant proteins (results not shown). Essentially identical results were obtained for cells transfected with the short variants (results not shown). Interestingly, a second band representing a molecular mass slightly lower than the main band was observed in all cells transfected with the FL constructs but not in the control. This band was often also seen in other experiments performed (see below). However, the identity of this band remains to be determined, but it probably represents a post-translationally modified or processed form of reggie-1-EGFP.

## Lipid modifications are necessary for the membrane association of reggie-1

To study the membrane association of the reggie-1-EGFP variants biochemically, a high-speed fractionation into membrane-associated (P) and soluble (S) components was performed with transfected HeLa cells. The fusion proteins were detected by Western-blot analysis of the corresponding fractions with monoclonal antibodies raised against reggie-1 or EGFP (Figure 3). The endogenous reggie-1 in non-transfected cells was detected with a monoclonal reggie-1 antibody and was found to be membrane-associated. The WT-FL and Cys4Ala-FL reggie-1-EGFPs were detected most often in the membrane fraction (P), although approx. 47 % of Cys4Ala-FL tended to be soluble (Figure 3 and Table 1). Interestingly, only the lower band of the double band in Cys4Ala-FL was found in the soluble fraction. Successive loss of further palmitoylation signals rendered the mutant variants Cys4 + 19Ala-FL and Cys4 + 19 +20Ala-FL more soluble and resulted in the appearance of the upper band in the soluble fraction. Most of the Gly2Ala-FL mutant (86 %) was detected in the soluble fraction, consistent with its solubilization by the missing myristoylation and palmitoylation signals. A fraction of the Gly2Ala-FL mutant protein still seemed to be capable

**Table 1 Summary of the properties of reggie-1-EGFP fusion proteins**

Quantification of the membrane association is based on three individual experiments and results are shown as S.D. Raft association is given as a mean for two individual experiments. n.d., not determined; Y, yes; N, no.

| Fusion protein | Myristoylated | Palmitoylated | % membrane associated | % raft associated | Oligomerized | Phenotype |
|---|---|---|---|---|---|---|
| WT-FL | Y | Y | 65 ± 10 | 62 | Y | Y |
| C4A-FL | Y | Reduced | 53 ± 12 | 55 | Y | Y |
| C4 + 19A-FL | Y | Residual | 25 ± 10 | 15 | Y | Y/N |
| C4 + 19 + 20A-FL | Y | N | 12 ± 5.9 | n.d. | Y | N |
| G2A-FL | N | N | 14 ± 5.9 | 18 | Y | N |
| WT-SH | Y | Y | 62 ± 2.1 | 2.0 | N | N |
| G2A-SH | N | N | 3.0 ± 2.0 | n.d. | N | N |
| NT | n.d. | n.d. | 63 ± 9.0 | n.d. | N | N |
| CT | n.d. | n.d. | 7.0 ± 3.2 | n.d. | Y | N |

of associating with membranes, as it was found in the pellet fraction. This fraction still remained associated with membranes, despite a high-salt/pH wash, which indicates a tight association of this fraction of the mutant protein with membranes (results not shown). The WT-SH reggie-1-EGFP variant was found to behave similarly to the WT-FL protein, whereas Gly2Ala-SH and other short mutants were virtually soluble (results not shown). We also tested the membrane association of the N-terminal half of reggie-1, which was found to be 63 % membrane-associated. Again, the lower band of a double band was found only in the soluble fraction.

### Homo-oligomerization of reggie-1 is mediated by the C-terminal half of the protein

Oligomerization of raft-associated proteins has been suggested to stabilize the association of acylated proteins with detergent-insoluble membranes [15]. To test if the reggie-1-EGFP fusion proteins are capable of oligomerization, sucrose-density-gradient fractionation of native proteins extracted from membranes with *N*-octylglucoside, which is known to disrupt rafts but to preserve protein oligomers, was performed. Endogenous reggie-1 was found to localize in fractions 6 and 7 which correspond to a molecular mass of approx. 70–150 kDa (dimers to trimers) and in fraction 10, indicative of higher-molecular-mass complexes (Figure 4A). The WT-FL reggie-1-EGFP was detected enriched in fraction 5, indicative of monomers, and in fractions 7–10, suggesting that it was capable of forming oligomers (Figure 4B). A very similar distribution was observed in the case of the Gly2Ala-FL mutant, which was found mostly in fractions 7–10 (Figure 4C). Interestingly, again a double band was seen the FL fusion proteins, the lower band being associated with the monomeric fractions, whereas the upper band was detected in the oligomeric complexes.

In contrast with the FL fusion proteins, the WT-SH protein was found to be concentrated in fraction 4, which corresponds to a molecular mass of 30 kDa (Figure 4D), in agreement with the monomeric form. All SH mutant variants were found to behave similarly (results not shown), demonstrating that the fusion proteins containing only the first 30 amino acids of reggie-1 are not able to assemble into oligomers.

The density-gradient method used above allows the detection of oligomeric complexes of proteins, but not the characterization of their oligomeric nature (homo-oligomers versus hetero-oligomers). To test if reggie-1 was capable of forming homo-oligomers, yeast two-hybrid analysis was performed. Reggie-1 and its N- and C-terminal halves were expressed in yeast *Saccharomces*

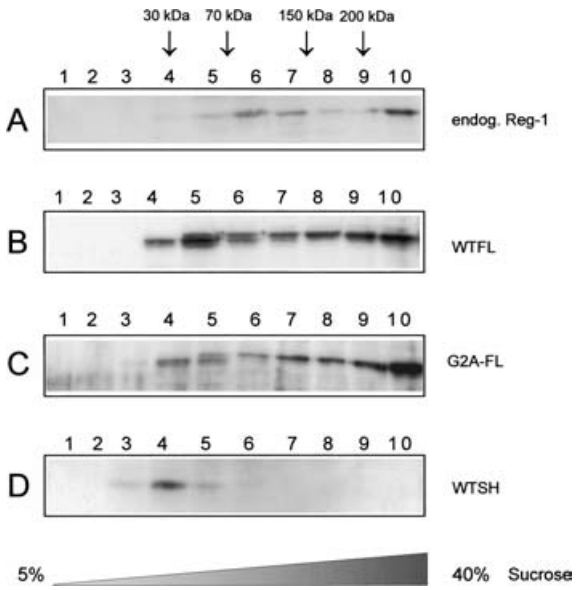


**Figure 4 Oligomerization of reggie-1**

Sucrose-density-gradient ultracentrifugation of native protein complexes from transfected HeLa cells was performed. FL fusion proteins were detected using a reggie-1-specific antibody, and truncated fusion proteins were detected using a GFP antibody. (**A**) Endogenous reggie-1 (47 kDa) was found in fractions higher than its monomeric molecular mass and in fractions representing high-molecular-mass oligomers. (**B**, **C**) Both the WT-FL and Gly2Ala-FL fusion proteins (74 kDa) were found in fractions 7–10 indicative of oligomers, in addition to the monomers in fractions 4–6. (**D**) The truncated WT-SH fusion protein (30 kDa) was only found in fraction 4, corresponding to its monomeric molecular mass.

*cerevisiae* as fusion proteins of the DNA-binding domain (BD) or activation domain (AD) of the Gal4 transcription factor and tested for interaction (Figure 5). FL reggie-1-BD was capable of interacting with FL reggie-1-AD and with the C-terminal half of reggie-1-AD. The C-terminal halves were also capable of interacting with each other, but not with the N-terminal half of reggie-1. No interactions could be detected using the N-terminal half of reggie-1 or a control protein (Lamin C). Identical results were obtained with both fusion domains (BD and AD), indicating that these interactions are independent of the fusion-protein context. The interactions of the constructs containing the FL sequence or the C-terminal half of reggie-1 were found to be very strong, as indicated by growth on selective media deficient in four nutrients (− adenine/− His/− Leu/− Trp) and blue staining [X-$\alpha$-Gal (5-bromo-4-chloroindol-3-yl $\alpha$-D-galactopyranoside reaction)] on $\alpha$-galactosidase assay on plates (Figure 5).

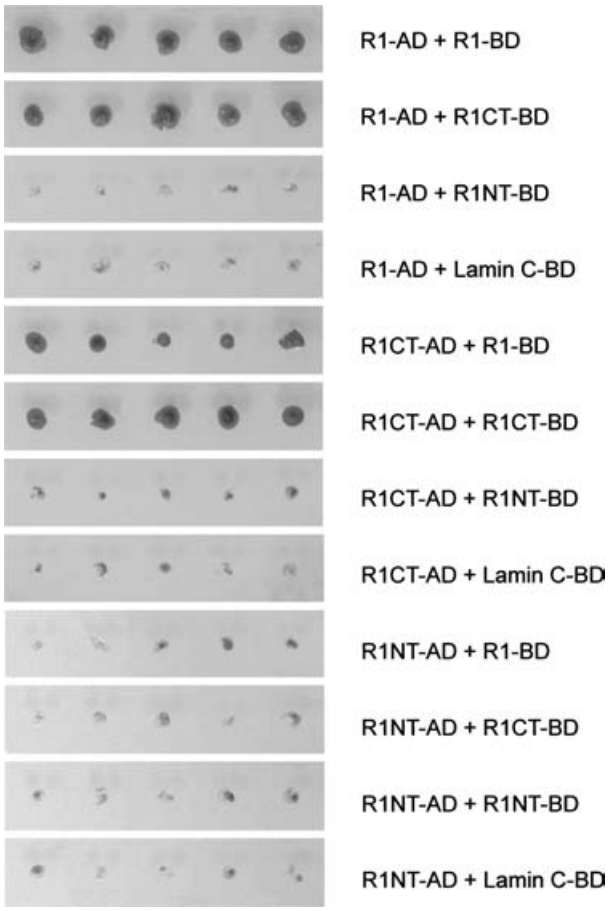


**Figure 5 Yeast two-hybrid analysis of the homophilic interaction and oligomerization of reggie-1**

The FL reggie-1 (R1) coding region or parts thereof (R1NT and R1CT) were cloned into two-hybrid vectors as fusion proteins with the Gal4 transcription factor binding (BD) or activating (AD) domain and tested for interaction in yeast two-hybrid assays. Interaction was monitored as growth on deficient media (− adenine/− His/− Leu/− Trp) containing X-$\alpha$-Gal. Strong interaction is indicated by growth and blue staining (X-$\alpha$-Gal reaction) of the colonies. Interactions were detected between all fusion proteins containing the C-terminal half of reggie-1 (R1 and R1CT) but not with the N-terminal half (R1NT). No interaction was seen with the control protein lamin C demonstrating the specificity of the interactions.

## Raft association of reggie-1 is mediated by lipid modifications and oligomerization

Since palmitoylation has been shown to mediate raft association of some proteins, detergent extraction and Optiprep density-gradient centrifugation ([20], with modifications as in [21]) were used to study the raft association of the reggie-1-EGFP fusion proteins. Western blots of the gradient fractions with reggie-1 or EGFP antibodies demonstrated that WT-FL reggie-1-EGFP (Figure 6A) was found to the most part floating in the light fractions 1 and 2, which have been shown to be enriched in rafts, and a small fraction was located in fraction 6. Similarly, the Cys4Ala-FL mutant (Figure 6B) was also detected in fractions 1 and 2, the remainder residing in fraction 6. Cys4 + 19Ala-FL (Figure 6C), Gly2Ala-FL (Figure 6D) and Cys4 + 19 + 20Ala (results not shown) were found enriched in fractions 5 and 6, although a small portion of Gly2Ala-FL was present in fraction 1. Interestingly, the WT-SH fusion protein (Figure 6E), which is both palmitoylated and myristoylated, was found to be enriched in the bottom-most non-raft fractions 5 and 6. This suggests that the lipid modifications in the WT-SH-reggie-1-EGFP are alone not sufficient to mediate the raft association of reggie-1, but additional determinants, such as oligomerization, need to be present for detergent insolubility.

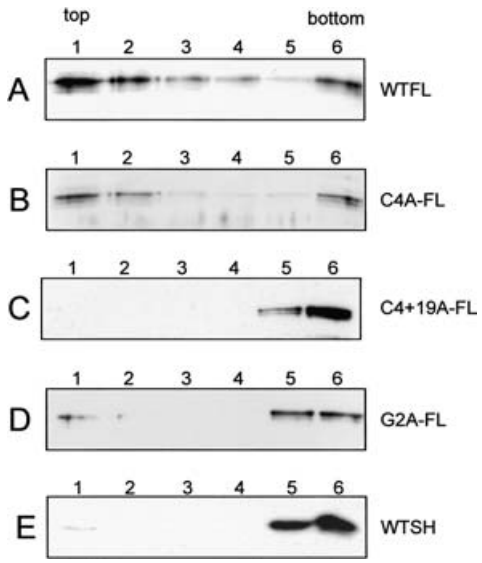


**Figure 6 Raft association of reggie-1: dependence on the lipid modifications**

Detergent-insoluble raft membranes from transfected HeLa cells were isolated by extraction with 1 % Triton X-100 at 4 °C and density-gradient ultracentrifugation. FL fusion proteins were detected using a reggie-1-specific antibody, truncated fusion proteins using a GFP-antibody. (**A**) The WT-FL fusion protein was enriched in the light fractions 1 and 2 and a smaller part was found in the bottom fraction 6, showing that this protein is raft-associated. (**B**) The Cys4Ala-FL fusion protein behaved very similar to WT-FL, although the degree of raft association was somewhat less. (**C**) Cys4 + 19Ala-FL mutant is found only at the bottom of the gradient (fractions 5 and 6). (**D**) Gly2Ala-FL fusion protein was strongly enriched in the bottom fractions 5 and 6, with only a very small amount of protein found in fraction 1, consistent with the large degree of solubilization of this mutant. (**E**) The WT-SH fusion protein, although myristoylated and palmitoylated, was not raft-associated, but enriched in fractions 5 and 6.

## Reggie-1 induces filopodia-like protrusions in several epithelial cell lines

Fluorescence analysis using a confocal laser-scanning microscopy of the WT-FL reggie-1-EGFP fusion protein in HeLa cells showed that it was localized in small patches at the plasma membrane and considerably enriched in structures at the cell periphery (Figure 7A). In addition, cells transfected with WT-FL fusion protein showed numerous fine filopodia-like protrusions of the plasma membrane, which could not be observed in control cells transfected with a plasma-membrane-associated YFP marker protein (Figure 7F). The protrusions induced by reggie-1 were shown to contain actin, which implies filopodial character (results not shown).

Effect of the mutant reggie-1-EGFP variants on the phenotype was also tested in HeLa cells. Cells transfected with the Cys4Ala-FL variant (Figure 7B) were found to exhibit the filopodial phenotype similarly to that induced by the WT-FL protein. Filopodia were also detected in cells expressing high amounts of Cys4 + 19Ala-FL mutant, although the cells seemed more elongated and a large fraction of the fusion protein was obviously soluble (Figure 7C). Low-expressing cells did not exhibit filopodia, indicating that the phenotype is dependent on the expression level. Neither of the soluble Cys4 + 19 + 20Ala or Gly2Ala-FL mutants (Figures 7D and 7E) was capable of producing the phenotype,

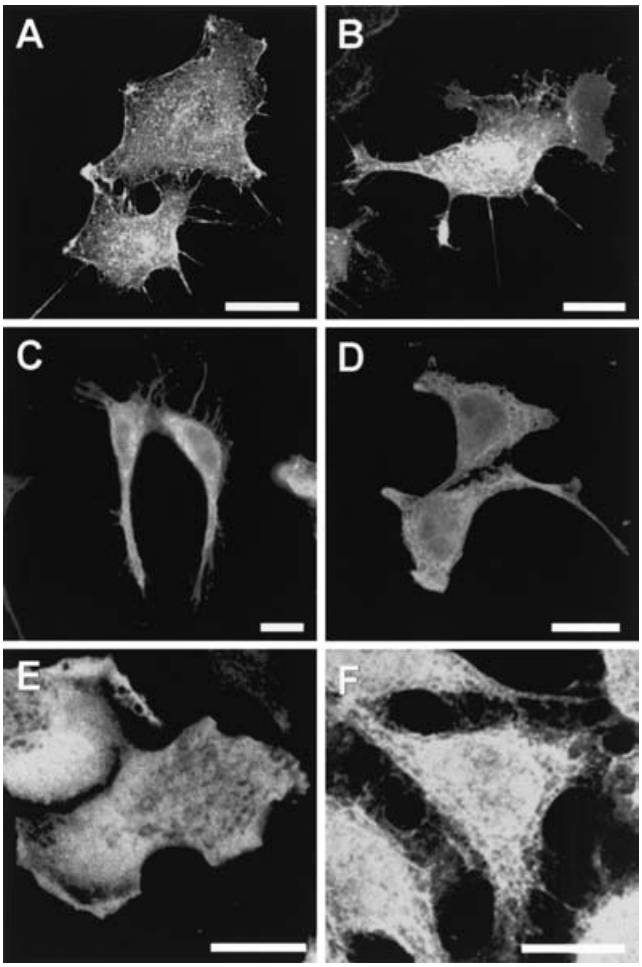


**Figure 7 Induction of a filopodial phenotype in various epithelial cell lines by overexpression of WT-FL- and Cys4Ala-FL-reggie-1-EGFP**

Cells were transiently transfected with the various reggie-1-EGFP fusion proteins or a plasma-membrane-associated YFP construct, and analysed by means of confocal laser scanning microscopy. HeLa cells transfected with WT-FL (**A**) or Cys4Ala-FL (**B**) show a patchy pattern at the plasma membrane and induction of numerous filopodia, which are also detected in cells over-expressing high amounts of Cys4 + 19Ala mutant (**C**), but not in low-expressing cells (results not shown). Filopodia were not detected in cells expressing Cys4 + 19 + 20Ala-FL (**D**), Gly2Ala-FL (**E**), or in control cells transfected with plasma-membrane-associated YFP (**F**). Scale bar, 10 $\mu$m.

indicating that membrane association is necessary for the induction of filopodia by overexpression of reggie-1.

To study if the reggie-1-EGFP is capable of inducing a similar phenotype in various epithelial cells, human keratinocytes (HaCaT cells, [18]) and rat thyrocytes (FRT cells) were transfected with the reggie-1-EGFP fusion construct. In both cell lines, a large number of filopodia were detected, due to overexpression of the WT-FL reggie-1-EGFP fusion protein (Figures 8A and 8B). These filopodia-like structures were often detected at the sites of cell–cell contacts (Figures 8C and 8D, HeLa cells), the reggie-1-EGFP protein being concentrated at the tips of these structures. Moreover, zipper-like structures were observed which exhibited a high accumulation of the reggie-1-EGFP fusion protein at sites of cell contacts.

To make sure that the observed phenotype is indeed caused by reggie-1 overexpression, we generated a muristerone-inducible expression system for reggie-1-EGFP in stably transfected CHO cells (Figures 9A and 9B). As a control, we used a CHO cell line

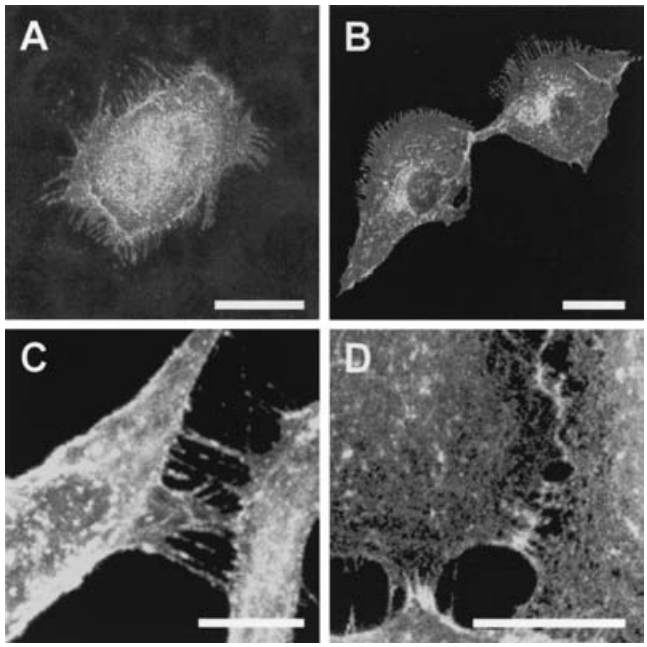


**Figure 8 Induction of filopodia in other cell types and association of reggie-1-EGFP in cell contact sites**

HaCaT (**A**) and FRT (**B**) cells transfected with WT-FL also show numerous filopodia. (**C**, **D**) In HeLa cells transfected with WT-FL, reggie-1 was frequently found enriched in contact sites between two neighbouring cells, often concentrated in zipper-like structures with numerous interdigitating filopodia. Scale bar: **A**, **B** and **D**, 10 $\mu$m; **C**, 5 $\mu$m.

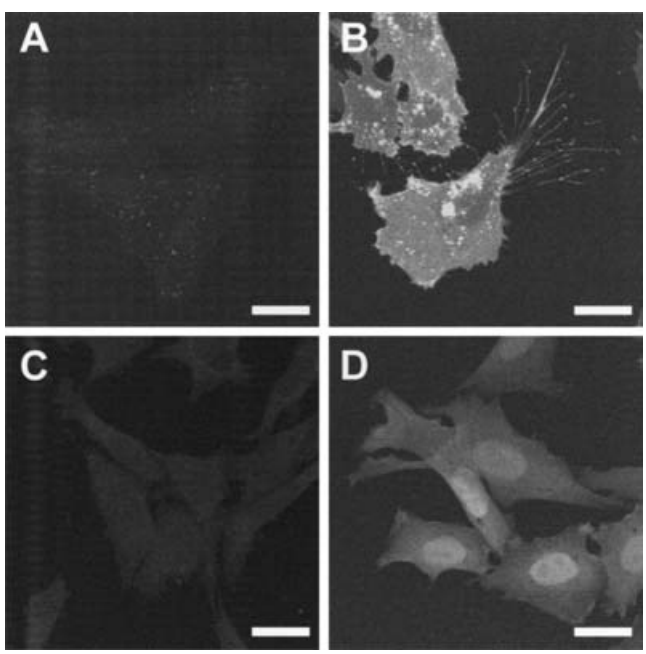


**Figure 9 Muristerone-induced expression of WT-FL-reggie-1-EGFP in CHO cells promotes filopodia formation**

CHO cells stably transfected with reggie-1-EGFP or EGFP-only were induced with muristerone and then analysed by means of confocal laser scanning microscopy. Fluorescence images from BGL236 cells with inducible expression of a reggie-1-EGFP fusion protein and BGL100 cells with inducible expression of EGFP are shown. (**A**, **B**) Reggie-1-EGFP-transfected CHO cells; (**C**, **D**) EGFP-transfected CHO cells; (**A**, **C**) uninduced; (**B**, **D**) induced. Note that induction of the reggie-1-EGFP fusion protein leads to changes in cell morphology with formation of filopodia, whereas expression of EGFP alone does not affect morphology of the cells. Scale bar, 10 $\mu$m.

overexpressing EGFP in an inducible manner (Figures 9C and 9D). Without induction, neither one of the proteins was expressed (Figures 9A and 9C). However, after inducible overexpression of reggie-1-EGFP, numerous filopodia were detected (Figure 9B), which could not be observed after induction of EGFP only (Figure 9D). This indicates that the observed filopodial induction is indeed dependent on reggie-1 overexpression in these cells.

## DISCUSSION

### Membrane association of reggie-1

Reggie-1 and -2 are ubiquitously expressed lipid raft proteins that have been linked with many signalling events such as insulin signalling, but their molecular function remains elusive. Despite high evolutionary conservation, reggies do not exhibit any recognizable domains or motifs that might give clues to their function. However, the N-terminal regions of both proteins show relatively high homology with the respective regions of stomatin and prohibitin, two proteins with relatively unknown *in vivo* functions. Thus this domain has been called SPFH (stomatin, prohibitin, flotillin homology) [22] or PHB (prohibitin homology; [13]) domain, but the function of this hypothetical domain is not known.

Recent findings from Morrow et al. [13] suggested that the membrane association of reggie-2 would be mediated by a single palmitoylation together with the hydrophobic regions in the PHB domain. However, this cannot be applied for reggie-1, which is different from the other members of the SPFH family in that it contains a putative myristoylation site in Gly-2, which is not present in other SPFH members. We have here shown that this site is indeed myristoylated in rat reggie-1 and plays a role in the membrane association of reggie-1. In addition, our results suggest that several palmitoylation sites can be utilized and the degree of palmitoylation, which is a reversible modification, regulates the membrane and even raft association of reggie-1 together with myristoylation.

Rat reggie-1 does not seem to contain any continuous, truly hydrophobic region that could form a membrane-anchoring structure. Two short stretches of hydrophobicity are present in the N-terminal region, which have been speculated to participate in membrane anchoring. However, it is highly unlikely that reggie-1 would possess a transmembrane domain. This is also supported by the fact that reggie-1 does not contain any signal-peptide-like sequence and is not accessible with antibodies or proteases in unpermeabilized cells [4,5]. Furthermore, the two residues, Cys-19 and Cys-20 at least one of which we have here shown to be palmitoylated, are located in the hydrophobic region, making it unlikely to span through the membrane. Our short construct with amino acids 1–30, which contains all the lipid modification sites and the two above-mentioned hydrophobic regions, was significantly more soluble than the FL protein and not capable of associating with rafts. Therefore we speculate that the hydrophobic region is not a major determinant for membrane and raft association, whereas the more C-terminal region of the protein seems to be important.

Although the WT-SH variant contains all the lipid modifications of reggie-1, it was found not to be capable of associating with lipid rafts, and its cellular localization was somewhat different from the WT-FL protein in that less of the protein seemed to be present at the plasma membrane and more intracellular, vesicular staining was observed. This is well in accordance with previous results from McCabe and Berthiaume [23], who also produced similar deletion constructs of many proteins known to associate with membranes/rafts by means of acylation. They could show that in many cases, acylation alone was not sufficient to target the GFP fusion proteins of the N-terminal part of the respective proteins to their expected subcellular localization and to rafts. They also speculated that further determinants such as oligomerization or protein–protein interactions are necessary to mediate raft association, a conclusion that can also be drawn for the WT-SH reggie-1 in this study. These findings also underscore the complexity of the mechanisms of raft association, and would suggest that the regulation of raft localization *in vivo* probably depends on various features of the proteins.

The C-terminal half of reggie-1 was here shown to be important for homo-oligomerization that in turn seems to enhance membrane and raft association, as suggested by the fact that the fully lipid-modified short protein, which does not oligomerize, is not raft-resident. Oligomerization has also been suggested for human erythrocyte reggie-1 [24] and was also speculated to play a role in the membrane association of reggie-2 [13]. Interestingly, we could also detect a heterophilic interaction between reggie-1 and -2 in our yeast two-hybrid assay that was also mediated by the C-terminal part of reggie-1 (our unpublished work). Hetero-oligomerization is also supported by the fact that reggies can be co-immunoprecipitated *in vitro* [5] and they partially co-localize in the same patch-like structures in neurons and haematopoietic cells [4,5,25]. The functional implications of hetero-oligomerization need to be experimentally addressed since many cell lines do not express detectable levels of reggie-2, whereas reggie-1 is abundant in all cell lines we have tested so far.

### Functional implications

Our results show that overexpression of reggie-1 results in the generation of numerous filopodia-like structures in several cell lines. Filopodia were also observed in our inducible expression system in CHO cells, indicating that this represents a direct effect of reggie-1 overexpression. Although the exact molecular function of reggie-1 still remains to be studied, our results strongly indicate a role for reggie-1 as a signalling protein capable of regulating multiprotein complexes involved in transmembrane signalling. The observed enrichment of the reggie-1 fusion proteins at the cell–cell contact sites and induction of filopodia, which are actin-dependent structures, suggest a link to the cytoskeleton. This is well in accordance with previous findings that have shown an enrichment of reggies in the filopodia of neuronal growth cones and in cell–cell contact sites of neurons and neuronal PC12 cells [4,5]. In epithelial cells, filopodia have been shown to mediate the initial contact between two adjacent cells [26] and they play a role in dynamic processes such as cell migration and morphogenesis of epithelia [27]. The direct functional association of reggie-1 with actin cytoskeleton, as shown in this study, also nicely supports the association of reggie-1 with molecules involved in cell adhesion. This was already suggested by the fact that the original cloning of reggies by two independent groups [1,28] took place by means of antibodies raised against cell-surface proteins playing a role in cell adhesion. However, later studies have shown that these antibodies actually do not recognize reggies directly, but their use rather results in co-immunoprecipitation of reggies with the true antigens such as cell-surface molecule Thy-1 [29].

A similar filopodial phenotype as shown here was also observed in an earlier study in COS cells after expression of the severely N-terminally truncated variant of reggie-1/flotillin-2 called epidermal surface antigen, fusioned N-terminally with GFP [30]. Whereas our results indicate that membrane association is necessary for the phenotype, Hazarika et al. [30] concluded

that the apparently soluble protein was capable of inducing a phenotype. Unfortunately, the authors did not analyse the degree of membrane association biochemically. Therefore it is possible that some of the fusion proteins might have been able to associate with the membranes by oligomerizing with the endogenous reggie-1 protein, resulting in a functional complex capable of inducing filopodia. We could show in the present study that the Cys4 + 19Ala-FL mutant protein, which is only 25 % membrane-associated, could induce a phenotype, but only when it was over-expressed in high degree at a single-cell level. However, the non-myristoylated and, thus, non-palmitoylated Gly2Ala-FL protein did not produce any phenotype even in high-expressing cells, although it is also 14 % membrane-associated. Although we cannot completely explain these discrepancies in the light of our present knowledge, it is possible that minor differences in the local concentration of reggie-1 oligomers and the capability of the mutant proteins to interact with further proteins necessary for the signalling might be responsible for these functional differences.

According to our results presented here, the lipid modifications of reggie-1 seem to play an important role in the function of the protein, as evidenced by the dependence of the phenotype induction on the lipid modifications and oligomerization. Although the capability of the WT and Cys4Ala protein to induce filopodia nicely correlated with raft association, this was not the case with Cys4 + 19Ala variant, which we could not detect in significant amounts in the raft fractions in our floating gradients after detergent extraction. However, this mutant was only capable of inducing a phenotype when expressed in high levels. Thus we cannot exclude that in these cells, some of the mutant protein might be associated with rafts, which would remain badly detectable in our gradients, which do not distinguish between expression levels in individual cells.

In addition to myristoylation and palmitoylation, other post-translational modifications might play a role in regulating the cellular localization and function of reggie-1 which contains, e.g., several putative phosphorylation sites. In addition to phosphorylation, a putatively proteolytic processing of reggie-1 on platelet activation has been described [31]. Thrombin activation of platelets resulted in a reggie-1 double band, the appearance of which could be inhibited with calpain inhibitors, indicating a role for calpain in the processing of reggie-1 after cellular activation. Interestingly, we also observed a double band in several experiments performed in this study, the lower band being specifically associated with the monomeric forms of reggie-1 found in the soluble pool. As the double band was also seen in the case of the N-terminal but not the C-terminal variant of reggie-1, the processing probably occurs near the N-terminus beyond lipid modifications, which would result in dissociation of the protein from the membranes. However, the significance of this proteolytic processing and the location of the processing site in reggie-1 remain to be determined, but it is possible that the processing results in an altered function and/or localization of the reggie-1 protein.

Taken together, in the light of our results and previous findings, a role for the reggie proteins, especially reggie-1, as signalling molecules in processes that result in actin reorganization seems plausible. However, the molecular details and the interaction partners that are necessary for these events remain to be described.

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