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Gonzalo P. Solis, Maja Hoegg, Christina Munderloh, Yvonne Schrock, Edward Malaga-Trillo, et al.. Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. *Biochemical Journal*, 2007, 403 (2), pp.313-322. 10.1042/BJ20061686 . hal-00478693

HAL Id: hal-00478693

<https://hal.science/hal-00478693>

Submitted on 30 Apr 2010

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Reggie/flotillin proteins are organized into stable tetramers in membrane-microdomains

Gonzalo P. Solis*, Maja Hoegg[§], Christina Munderloh, Yvonne Schrock, Edward Malaga-Trillo,
Eric Rivera-Milla, and Claudia A.O. Stuermer

Department of Biology, University of Konstanz, 78467 Konstanz, Germany

*To whom correspondence should be addressed (email Gonzalo.Solis@uni-konstanz.de)

[§]Present address: Department of Biochemistry and Molecular Biology, University of Calgary, 3330
Hospital Drive NW Calgary, Alberta, Canada T2N 4N1

Short title: Tetramerization and stabilization of reggie/flotillin proteins

SYNOPSIS

Reggie-1 and -2 proteins (flotillin-2 and -1, respectively) form their own type of non-caveolar membrane microdomains, which are involved in important cellular processes such as T cell activation, phagocytosis, and signaling mediated by the cellular prion protein and insulin; this is consistent with the notion that reggie-microdomains promote protein assemblies and signaling. While it is generally known that membrane-microdomains contain large multiprotein assemblies, the exact organization of reggie-microdomains remains elusive. Using chemical cross-linking approaches, we demonstrate here that reggie-complexes are composed of homo- and hetero-tetramers of reggie-1 and -2. Moreover, native reggie-oligomers are indeed quite stable, since uncross-linked tetramers are resistant to 8 M urea treatment. We also show that oligomerization requires the C-terminal but not the N-terminal halves of reggie-1 and -2. Using deletion constructs, we analyzed the functional relevance of the three predicted coiled-coil stretches present in the C-terminus of reggie-1. We experimentally confirmed that reggie-1 tetramerization is dependent on the presence of coiled-coil 2 and, partially, of coiled-coil 1. Furthermore, since depletion of reggie-1 by siRNA silencing induces proteasomal degradation of reggie-2, we conclude that the protein stability of reggie-2 depends on the presence of reggie-1. Our data indicate that the basic structural units of reggie-microdomains are reggie homo- and hetero-tetramers, which are dependent on the presence of reggie-1.

Key words: Reggie/flotillin, oligomerization, cross-linking, membrane microdomains, lipid rafts

INTRODUCTION

Reggie-1 and -2 (flotillin-2 and -1, respectively) are two highly conserved lipid raft-associated proteins, which form their own type of non-caveolar microdomains [1,2], and define a novel clathrin-independent endocytic pathway in mammalian cells [3]. Reggies were first discovered as proteins upregulated in retinal ganglion cells during axon regeneration in fish and rats [4,5]. They were independently identified as proteins contained in the “floating” lipid raft fraction from mouse lung tissue, and called flotillin-1 and flotillin-2 [6]. To date, reggie proteins have been identified in all metazoans, and reggie-like proteins in bacteria, fungi and higher plants [7,8]. Reggies and reggie-like proteins belong to the so-called SPFH¹ (Stomatin/Prohibitin/Flotillin/HflK/C) protein superfamily, whose members share a common N-terminal domain of unknown function, the SPFH domain [9]. In contrast to other SPFH proteins, the C-terminus of reggies is longer and includes a so-called “flotillin” domain, which is characterized by the presence of repeats rich in glutamate and alanine, which are predicted to form three coiled-coil stretches [6,8,10].

Even though the functions of reggie proteins remain poorly understood, they have been implicated in interesting cellular processes, such as insulin signaling [11], phagocytosis [12], axon growth [4,5], and lymphocyte activation [13-17]. For instance, in Jurkat T cells, reggies assemble into a preformed cap which recruits all the known signaling molecules involved in activation [13-16]. Using the same cells, we have shown that antibody-mediated signaling of the cellular prion protein takes place in the preformed reggie cap region [17]. During zebrafish development, knock-down of reggies produces severe morphological defects (Malaga-Trillo *et al*, unpublished). While knock-out of reggie proteins in *Drosophila* does not produce evident phenotypes, overexpression of reggie-1 and -2 in the eye imaginal disc leads to severe mistargeting of cell adhesion molecules, resulting in irregular ommatidial patterns [18]. Moreover, abnormal *wingless* signaling and wing development can be observed upon reggie overexpression [18]. Hence, various lines of evidence support the notion that reggie-microdomains form scaffolds for protein assemblies, which are involved in signaling across the plasma membrane [19].

That reggie proteins form membrane-microdomains is evidenced by their distinct punctated distribution along the plasma membrane, and further supported by our own electron microscopy analyses defining reggies as non-caveolar clusters of a surprisingly constant average size of $\leq 0.1 \mu\text{m}$ [13]. However, it is not known how reggie proteins are organized within these microdomains, and the little experimental data available disagree about the exact size of reggie-complexes [20,21]. Here we determined the molecular size of oligomers of endogenous and exogenous reggie proteins by chemical cross-linking experiments in living cells. Our goal was to obtain direct information on the stoichiometry of reggie-complexes, and to define the reggie domains required for oligomerization. We demonstrate that reggie proteins form homo- and hetero-tetramers via their C-terminal regions. Moreover, we also tested the oligomerization abilities of the three coiled-coil stretches predicted at the C-terminal half of reggie-1. In addition, siRNA experiments

reveal that reggie-2 protein undergoes proteasomal degradation upon silencing of reggie-1. Our studies show that reggie-1 and -2 are organized as homo- and hetero-tetramers at the plasma membrane, where the presence of reggie-1 is required for the stabilization of reggie-2. Here we have uncovered the basic building blocks of reggie-microdomains, providing new insight into their formation and stabilization dynamics. Clarifying these processes is pivotal to better understand the function of reggie-1 and -2 as signaling platforms.

EXPERIMENTAL

Reagents and antibodies

Cell culture reagents were purchased from GIBCO BRL. Antibodies were purchased from the following companies: monoclonal antibody (mAb) α -Flotillin-2/ESA (reggie-1) and mAb α -Flotillin-1 (reggie-2) from BD Biosciences, mAb α -GAPDH (6C5) from Abcam, and mAb α -HA (3F10) and mAb α -GFP (7.1/13.1) from Roche. Polyclonal antibody (pAb) α -GFP from Clontech, pAb goat α -rabbit Alexa-488, pAb donkey α -rat Cy-3, pAb goat α -rabbit HRP, and pAb goat α -mouse HRP from Jackson ImmunoResearch, and pAb goat α -rat HRP from Pierce Chemical. The pAb α -NCAM was kindly provided by E. Bock (University of Copenhagen, Denmark). Disuccinimidyl suberate (DSS), *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), Dimethyl suberimidate (DMS) and Disuccinimidyl tartarate (DST) were purchased from Pierce Chemical. Lactacystin was kindly provided by M. Groettrup (University of Konstanz, Germany). All other reagents were purchased from Sigma-Aldrich.

Cell culture and transfection

N2a cells were maintained in MEM containing 10% fetal calf serum, L-Glutamine, Pyruvate and Penicillin-Streptomycin at 37°C and 5% CO₂, and grown on poly-Lysine coated coverslips (for immunofluorescence studies) or in 6-well plates (for cross-linking experiments) for 24 h prior to transfection. N2a cells were transiently transfected with the relevant vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations, and experiments were performed 20 h post transfection.

Cloning

Vectors including the rat cDNA sequences of reggie-1 and -2 fused to EGFP were generously provided by R. Tikkanen (University of Frankfurt, Germany). For cloning HA- and BiFC-tagged constructs (Figure 1), reggie cDNAs were amplified by PCR from the reggie-EGFP vectors described above using *Pwo* DNA polymerase (Peqlab Biotechnologie). N-terminal HA-tagged constructs were obtained by cloning the

reggie sequences into the EcoRI and XhoI sites of pJG4-5 vector (Invitrogen). Then, the HA-tagged reggie constructs were amplified by PCR and cloned into the EcoRV site of the pcDNA3 vector (Invitrogen). C-terminal HA-tagged reggie-1 and -2 were obtained by PCR with primers including the HA-tag, and finally cloning into the BamHI and XhoI sites of pcDNA3 vector (Invitrogen). BiFC-reggie proteins were obtained by cloning in frame the reggie sequences into the EcoRI and XhoI sites of pBiFC-YC155 vector (kindly provided by M. D'Silva; University of Konstanz, Germany) [22]. These vectors coding for reggie proteins fused to the C-terminal half of the YFP (from amino acid 155 to 238).

Cross-linking experiments

Transiently transfected and non-transfected N2a cells were washed twice with PBS. Chemical cross-linkers (in DMSO) were applied to the cells at the final concentrations indicated in the figure legends. After incubation at RT for the time indicated, cells were directly boiled in SDS-PAGE sample buffer. The resulting extracts were analyzed by immunoblotting with mAbs specific for reggie-1 or -2, mAbs against the HA epitope or the GFP protein, and pAb against GFP.

Urea treatments

Non-transfected N2a cells were washed twice with PBS and lysed in PBS supplemented with the urea concentrations indicated in the corresponding figure. After 1 h of incubation at RT, the resulting extracts were run into a 10% SDS-PAGE and analyzed by immunoblotting with a mAb specific for reggie-1. Additionally, the 6 M urea cell extract was analyzed using a 10% SDS-PAGE supplemented with 6 M urea followed by Western blotting for detection of reggie-1.

High-speed cell fractionation experiments

Cell extracts of transiently transfected N2a cells were prepared using non-denaturing conditions and fractionated using high-speed centrifugation. Briefly, N2a cells were washed twice with PBS and resuspended in hypoosmotic buffer (20 mM HEPES pH 7.0). The cell suspension was passed through a 27 Gx3/4" needle 20 times, and nuclei and unbroken cells were removed by 10 min centrifugation at 1000 x g and 4°C. Cytosolic and membrane fractions were separated by 60 min centrifugation at 100,000 x g at 4°C. Fractions were directly boiled in SDS-PAGE sample buffer and analyzed by Western blotting using mAb against the HA epitope.

Western blot analysis

For SDS-PAGE and immunoblots [23], samples containing reggie species were separated on 10 or 15% gels under reducing conditions. As molecular marker the Precision Plus protein standard purchased from Bio-Rad Laboratories was used. After gel electrophoresis, proteins were transferred to Hybond-C Extra

nitrocellulose membranes (Amersham Biosciences) in a tank blot apparatus. The membranes were blocked in PBS containing 5% nonfat dried milk (1 h, RT) and incubated with the relevant Abs, defined above and below, in blocking solution for 1 h at RT or overnight at 4°C. After four washes in PBS, the blots were incubated with HRP-conjugated goat α -mouse, goat α -rat or goat α -rabbit pAbs in blocking solution for 1 h at RT, and, after extensive washing, developed with the use of the enhanced chemoluminescence substrate SuperSignal (Pierce Chemical) and Hyperfilm ECL (Amersham Biosciences).

Immunofluorescence

Transiently transfected N2a cells were fixed using 4% paraformaldehyde in PBS for 10 min, permeabilized in PBS containing 0.1% TX-100 for 1 min at RT and blocked with 1% BSA in PBS. Samples were exposed to the primary antibodies for 1h at RT, washed three times with PBS, and the secondary antibodies were applied for 1 h at RT. After several rinses with PBS, the cells were embedded in a mixture of 5 g Mowiol 4-88 (Hoechst), 20 ml PBS and 10 ml glycerol. Light microscopical analyses were performed with a confocal laser-scanning microscope (LSM 510, Carl Zeiss) equipped with a Plan-Apochromat $\times 63$ oil immersion objective (n.a.=1.4). Images were acquired with the LSM 510 software and further processed using Corel PHOTO-PAINT 11.

RNA interference

Small interfering RNA (siRNA) duplexes R1.0 and R2 were obtained from Dharmacon (Dharmacon, Lafayette, USA) and R1.1 from Ambion (Ambion, Huntingdon, UK). The target sequences for reggie-1 were 5'-GTTCATGGCAGACACCAAG-3' (R1.0) and 5'-GGTGAAGATCATGACGGAG-3' (R1.1) and 5'-CACACTGACCCTCAATGTC-3' (R2) for reggie-2. A commercial siRNA against luciferase of firefly (*Photinus pyralis*), referred to as GL2 (Dharmacon, Lafayette, USA), was used as a non-specific control. N2a cells were grown in 24-well plates and transfected with duplexed siRNAs (7.5 pmol/well) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Lactacystin was used at a final concentration of 10 μ M 8 h post transfection to block the 20 S subunit of the proteasome [24]. Cells treated or non-treated with lactacystin were lysed 48 h after transfection for Western blot analysis using GAPDH, reggie-1 and -2 specific mAbs. Total RNA extraction of the samples was obtained 48 h after transfection using the RNeasy Mini Kit (Qiagen). Then, samples were analyzed by RT-PCR using the primers R1-For 5'-CAGGTGAAGATCATGACG-3', R1-Rev 5'-ACCACAATCTCATCGAC-3', R2-For 5'-CTTGTGGCCCAAATGAG-3', and R2-Rev 5'-ATCTCCTGCTCCTGCAC-3'. As control were used the primers GAPDH-For 5'-GGTCGGTGTGAACGGATTTGGC-3' and GAPDH-Rev 5'-GGAGGCCATGTAGGCCATGAGG-3' designed to amplify a fragment of the GAPDH cDNA.

RESULTS

Reggie-1 and reggie-2 form homo- and hetero-tetramers

To examine the oligomerization states of endogenous reggie-1 and -2, we took advantage of DSS, a chemical compound which cross-links adjacent lysine residues within a range of 11.4Å. DSS-treated N2a cells were analyzed by Western blot of denatured cell extracts using mAbs specific for reggie-1 and -2, respectively (Figure 2). In addition to the known ~47 kDa reggie-1 and -2 monomers [5], we observe strong bands of ~200 kDa, corresponding to the expected size of reggie homo- and/or hetero-tetramers. Surprisingly, no bands corresponding to reggie dimers or trimers are observed even when DSS is applied for short times (1 min; Figure 2) or at lower concentrations (50 µM; Supplementary Figure 1A). Instead, as DSS treatment time increases, additional bands of even higher molecular weight become gradually more visible at the expense of monomer and tetramer species (Figure 2). These bands might correspond to larger complexes generated by the covalent linkage of two or more reggie-tetramers, though the participation of others proteins cannot be excluded. Identical results using DSS in PC12, HeLa and Jurkat T cells (results not shown) point to the widespread existence of reggie-tetramers. The existence of the ~200 kDa band was corroborated using other cross-linkers covering different distance ranges and amino acid specificities, such as DMS or MBS (Supplementary Figure 1B and C). The ~200 kDa bands are not observed upon treatment with DST, a DSS-analog with a shorter distance range (6.4Å), indicating that these bands represent the covalent binding of reggie proteins by their exposed lysine residues at a specific distance (results not shown).

To rule out that the complexes observed are induced by experimental cross-linking, we tested for the formation of stable reggie-oligomers in the absence of chemical cross-linkers and under increasing urea concentrations. Therefore, urea-treated N2a cell extracts were analyzed by Western blot using a specific reggie-1 mAb (Figure 3A). Notably, oligomers of ~200 kDa containing reggie-1 were observed even at 8 M urea, indicating that putative reggie-tetramers are very stable. Furthermore, comigration of the cross-linked and urea-resistant reggie-complexes at ~200 kDa strongly suggests that these complexes are identical. The oligomers detected here represent reggie-tetramers and not aberrantly migrating dimers or trimers, since 6 M urea cell extracts revealed the same ~200 kDa band when run under strong denaturing conditions (SDS-PAGE supplemented with 6 M urea; Figure 3A). Hence, endogenous reggies exist in a monomer-tetramer equilibrium.

To gain insight into the stoichiometry and the exact composition of reggie-oligomers, we performed cross-linking experiments on exogenously expressed reggie-1 and -2. We applied DSS to N2a cells transiently transfected with reggie-EGFP or -BiFC vectors, resulting in the expression of fusion proteins of easily discernable sizes. Besides the endogenous reggie-monomer and tetramer bands described above, we observed additional bands of ~75 and ~300 kDa, corresponding to the expected sizes of the monomeric

and homo-tetrameric reggie-1 or -2 EGFP fusion proteins (Figure 3B). Accordingly, DSS-treated cells expressing reggie-1 or -2 BiFC fusion proteins yielded additional bands of ~60 and ~240 kDa (Figure 3B), corroborating the existence of reggie-1 or -2 as monomers and homo-tetramers. To confirm that these additional bands contained reggie-EGFP and -BiFC fusion proteins, we reprobated the samples with a pAb against all fusion proteins, and we obtained the same bands (Supplementary Figure 2). These results strengthen the notion that the ~200 kDa bands observed upon DSS cross-linking or urea denaturation are indeed reggie-tetramers.

Although interactions between reggie-1 and -2 have been well studied [13,17,20], we did not observe the formation of reggie hetero-tetramers between endogenous and EGFP- or BiFC-reggies. The most likely explanation for this is that homo-tetramerization of reggie fusion proteins is favored over hetero-tetramerization with endogenous reggies because of their faster de novo synthesis. To further clarify this, we co-transfected N2a cells with vectors encoding differentially tagged reggie-1 and -2. Interestingly, DSS treatment of cells co-expressing reggie-1-EGFP (~75 kDa) and reggie-2-HA (~50 kDa) or reggie-1-HA (~50 kDa) and reggie-2-EGFP (~75 kDa) revealed the presence of an additional band of ~250 kDa (Figure 4), corresponding to the expected sizes of a hetero-tetramer formed by two molecules of each reggie. We also observed the reggie-EGFP homo-tetramers bands described above (~300 kDa; Figure 3B). Reggie hetero-tetramers of ~250 kDa were observed using cells transfected with different amounts of vector combinations (results not shown), and by reprobating the blots with mAbs against GFP and the HA-epitope, respectively (Supplementary Figure 3). As expected, homo-tetramers of HA-tagged reggie proteins migrate as ~200 kDa bands similar to the endogenous reggie-tetramers (Supplementary Figure 3). Taken together, our results show that reggie-1 and -2 build homo- and hetero-tetramers in living cells, and that hetero-tetramers are formed by two molecules of reggie-1 and -2.

Mapping of reggie oligomerization domains

To establish whether reggie-tetramerization depends on the presence of the SPFH or “flotillin” domains, cross-linking experiments were performed on N2a cells expressing three distinct truncated forms of HA-tagged reggies (Figure 1). These included one construct lacking the N-terminus implicated in membrane-association (HA-R1₄₁₋₄₂₈ and HA-R2₃₆₋₄₂₈), and the other two additionally lacking the “flotillin” (HA-R1₄₁₋₁₈₃ and HA-R2₃₆₋₁₇₉) or SPFH domains (HA-R1₁₈₄₋₄₂₈ and HA-R2₁₈₀₋₄₂₈). As shown in Figure 5A and B, HA-tagged reggies lacking only the N-terminal end are seen as monomers, tetramers and higher order oligomers, similar to full-length reggies (Figure 2 and 3). However, oligomerization of these constructs seems to be weaker than their full-length counterparts, suggesting that formation of reggie-tetramers is facilitated by association to cell membranes. In addition, constructs lacking the SPFH domain are able to form comparable multimers, although tetramers are no longer the dominant forms (Figure 5A and B). On the other hand, HA-reggie-1 and -2 constructs lacking the “flotillin” domain do not form oligomers but

appear as monomers instead (Figure 5A and B). Thus, our results indicate that the C-terminal half of reggies is necessary and sufficient for oligomerization, whereas the SPFH domain is dispensable.

It has previously been established that acylation and hydrophobic regions determine membrane and raft association of reggie-1 and -2 [20,25]. Since our constructs containing only C-terminal half are able to form oligomers, we tested whether they can indirectly associate to the plasma membrane via homo- and hetero-interactions with endogenous reggies. We examined the membrane association of the HA-tagged reggie constructs described above (Figure 1). Indeed, HA-reggie constructs lacking the residues for acylation and the hydrophobic domains (HA-R1₄₁₋₄₂₈ and HA-R2₃₆₋₄₂₈) can partially associate with the plasma membrane, as seen by colocalization with the cell surface protein NCAM (Figure 6A and D) and their presence in membrane fractions (Figure 7A and B). Therefore, endogenous reggies must be able to interact with these constructs and recruit them to the plasma membrane. Consequently, HA-reggie constructs including only the C-terminal half (HA-R1₁₈₄₋₄₂₈ and HA-R2₁₈₀₋₄₂₈) are partially associated with the plasma membrane (Figure 6C and F; Figure 7A and B). In the case of reggie-2, it is also possible that a second hydrophobic stretch predicted in the SPFH domain (residues 134 to 151; Figure 1) directly interacts with the cell membrane. Accordingly, the HA-R2₃₆₋₁₇₉ construct containing only the reggie-2 SPFH domain colocalizes with NCAM (Figure 6E) and is present in the membrane fraction (Figure 7B); this is not the case for the corresponding reggie-1 construct (Figure 6B and 7A). Hence, our data show that the C-terminal halves mediate reggie oligomerization, and that the SPFH domains of reggie-1 and -2 differ in their ability to associate with the plasma membrane.

Different coiled-coil regions in the C-terminal “flotillin” domain of reggie-1 are involved in tetramerization

Since our experiments showed that the C-terminal half is required for reggie tetramerization, we mapped this property more precisely. As mentioned above, the C-terminal half of reggies contains a so-called “flotillin” domain, which is predicted to form three adjacent coiled-coils (here termed coiled-coil 1, 2 and 3; Figure 1). To assess the specific contribution of these stretches towards tetramerization, we analyzed N2a cells expressing HA-tagged reggie constructs lacking the N- and C-terminal ends and one or more of the predicted coiled-coil stretches (Figure 1). Because reggie-1 proteins are more stably expressed than their reggie-2 counterparts (see below), we focused our analyses on the coiled-coil regions of reggie-1. As expected, the reggie-1 construct containing all three coiled-coil stretches (~37 kDa; HA-R1₄₁₋₃₅₃) forms tetramers upon DSS cross-linking (~150 kDa; Figure 5C), indicating that the entire coiled-coil region is necessary and sufficient for tetramerization. Since a construct lacking coiled-coil 3 (~33 kDa; HA-R1₄₁₋₃₂₁) retains the ability to form tetramers (~130 kDa; Figure 5C), we conclude that tetramerization does not require the presence of this stretch. Notably, the construct lacking only coiled-coil 2 (~27 kDa; HA-R1_{41-238/321-353}) does not form tetramers (Figure 5C), revealing that this stretch is essential for tetramerization.

Interestingly, this construct displays a residual ability to oligomerize, since a band corresponding in size to a trimeric species (~80 kDa) becomes visible (Figure 5C). Such a phenomenon is not observed for the construct lacking only coiled-coil 3 (HA-R1₄₁₋₃₂₁), indicating that this additional ability to oligomerize must be encoded in coiled-coil 1. Accordingly, the construct containing only coiled-coil 1 (~24 kDa; HA-R1₄₁₋₂₃₈) assembles into oligomeric species corresponding in size to trimers (~70 kDa). To confirm that the coiled-coil 1 construct has residual oligomerization abilities, we tested whether its interaction with endogenous reggies would result in its association with cellular membranes. As shown above (Figure 7A), the control construct lacking all coiled-coil stretches (HA-R1₄₁₋₁₈₃) is not significantly present in the membrane fraction, whereas the construct containing only coiled-coil 1 (HA-R1₄₁₋₂₃₈) clearly associates with the cell membrane (Figure 7C). Taken together, these results show that the ability of reggie-1 to tetramerize is conferred by the presence of coiled-coil 2 and, partially, of coiled-coil 1.

Reggie-2 undergoes proteasomal degradation in the absence of reggie-1

It has been reported in *Drosophila* mutants, that the protein stability of reggie-2 requires the expression of reggie-1 [18]. Similarly, we observe that our reggie-2 constructs are not as efficiently expressed in N2a cells as our reggie-1 constructs. To clarify this phenomenon, we analyzed the stability of reggie-2 upon siRNA-mediated gene silencing of reggie-1 in N2a cells. When cells are transfected with reggie-1 or reggie-2 siRNAs, the corresponding mRNA (Figure 8A) and protein levels (Figure 8B) are significantly reduced 2 days after transfection; control siRNAs produce no effect. Interestingly, transfection with reggie-1 siRNAs leads to depletion of both reggie-1 and -2 proteins (Figure 8B). RT-PCR analysis shows that reggie-1 siRNAs induce degradation of reggie-1 mRNA whereas the reggie-2 transcripts are not affected (Figure 8A). Therefore, the regulation of reggie-2 by reggie-1 occurs at the protein level. On the other hand, cells treated with reggie-2 siRNA lose the corresponding mRNA (Figure 8A) and protein (Figure 8B), but reggie-1 mRNA and protein remain unaffected. We observed identical effects using the same siRNAs in HeLa cells (results not shown).

Since the proteasome system is a central component for the degradation of intracellular proteins [26], we studied its participation in the degradation of reggie-2 after reggie-1 depletion. Notably, downregulation of reggie-2 protein by reggie-1 siRNA is no longer observed when N2a cells are incubated with 10 μ M of the 20 S proteasome inhibitor lactacystin, indicating that reggie-2 degradation occurs via the proteasome system (Figure 8B). These results reveal that the presence of reggie-1 is necessary for the stability of reggie-2, which otherwise undergoes proteasomal degradation.

DISCUSSION

Altogether, we demonstrate here that the organization of reggie-microdomains at the plasma membrane requires reggie protein interactions and involves the formation of reggie homo- and hetero-tetramers. We molecularly dissected the domains involved in the oligomerization process, and identified a coiled-coil stretch required for tetramerization. In addition, we show that reggie-2 undergoes proteasomal degradation in the absence of reggie-1.

Reggie proteins form homo- and hetero-tetramers in living cells

To date, the little evidence available on oligomerization of reggie proteins is vague, contradictory, and relies solely on sucrose gradient centrifugation methods. For instance, in HeLa cells, reggie-1 localizes in fractions of 70-150 kDa [20], whereas in human erythrocytes, both reggie proteins mainly localize in fractions of 300-400 kDa [21]. On the other hand, molecular interactions between reggie-1 and reggie-2 have been examined using yeast two-hybrid assays, co-immunoprecipitation, and co-localization [13,17,20]; however, no evidence for the formation of reggie hetero-oligomers has been provided so far. We assessed the oligomerization state of reggie proteins using chemical cross-linking, which is a powerful method to examine protein-protein interactions in living cells [27,28]. As reggies contain many lysine residues (34 and 30 in reggie-1 and -2, respectively), we used cell-permeable lysine-specific cross-linkers. Different homo- and hetero-bifunctional cross-linkers (DSS, MBS and DMS) with spacer arms between 12 and 9.9Å consistently demonstrate that endogenous reggies are present as monomers and tetramers in N2a cells as well as in other cell types (PC12, HeLa and Jurkat T cells). We did not observe reggie-tetramers using DST, a lysine-specific homo-bifunctional cross-linker with a short spacer arm (6.4Å), confirming the specificity of the cross-linking approach. We corroborated the existence of native reggie-tetramers by treating N2a cells with different concentrations of urea in the absence of cross-linkers. Interestingly, urea treatments did not reveal intermediate species such as dimers and trimers of full-length reggies, indicating that reggies exist in a monomer-tetramer equilibrium, as it has been reported for other coiled-coil containing proteins such as dynamin [29,30]. In fact, the reggie-oligomers observed are formed by very stable, high-affinity protein-protein interactions, which are resistant to 8 M urea. Moreover, the identical migration patterns of reggie-complexes in strong denaturing urea-gels support our view that cross-linked reggie-oligomers represent tetramers and not aberrantly migrating dimers or trimers, as has been shown for SDS-resistant SNARE-complexes [31]. Since our current structural model for reggie-monomers suggests that the C-terminal “flotillin” domain of both proteins is strongly stabilized via intramolecular interactions [8], we speculate that the only thermodynamically stable reggie-oligomerization state is the tetramer (possibly formed by a four-stranded parallel coiled-coil rod). Thus, intermolecular interactions between the coiled-coil regions might drive the monomer-tetramer

equilibrium, but not the formation of dimers or trimers. Intriguingly, we did not observe reggie intermediate species even at very low concentrations of homo- or hetero-bifunctional cross-linkers. Given the stochastic nature of chemical cross-linking, one might expect to find such intermediate species at low concentrations of cross-linker. However, the large number of lysine residues in the oligomerization region of reggie-1 and -2 might require even smaller titration intervals than the ones we used to obtain dimers and trimers. The confirmation that reggie-1 and -2 form homo- and hetero-tetramers was obtained by exogenous expression in N2a cells of EGFP- and BiFC-constructs as well as HA-tagged reggie proteins. We observe that cytosolic (truncated) reggie constructs oligomerize less efficiently than full-length proteins, suggesting that membrane association might facilitate reggie-tetramerization. Taken together, our results indicate that reggie proteins are only present in a monomer-tetramer equilibrium, and that reggie hetero-tetramers are built only by two molecules of each reggie under our experimental conditions; more data will be required to better understand this restricted stoichiometry. Thus, the reggie microdomains of living cells are formed by homo- and hetero-tetramers of reggie-1 and -2.

The C-terminal halves are responsible for tetramerization of reggie-1 and -2

Previous findings by Neumann-Giesen *et al.* had suggested that the C-terminal half of reggie-1 is important for homo- and hetero-interactions with reggie-2 [20]. Using DSS-based cross-linking experiments, we have now established that the C-terminal halves of reggie-1 and -2 are necessary and sufficient for tetramerization, whereas the SPFH domains are not required for oligomerization. Moreover, we show that, within the “flotillin” domain of reggie-1 (residues 239 to 321), coiled-coil 2 is necessary for tetramerization, and coiled-coil 1 (residues 184 to 238) also contributes partially to oligomerization. Since expression of reggie constructs containing only C-terminal halves form a wide continuum of multimers, it is possible that the SPFH domains restrict the oligomerization process specifically towards the formation of tetramers. In agreement with this view, expression of constructs containing the SPFH domain and either the C-terminal half or the “flotillin” domain indeed forms only tetramers. Because of the sequence similarity between reggie-1 and -2 at the C-terminal half [8], we expect that coiled-coil 2 of reggie-2 might likewise be involved in tetramerization, a point which requires further analysis. Since our data clearly demonstrate that the C-terminal domains of reggies mediate oligomerization, we propose the term “oligomerization domain” instead of “flotillin domain”, which was based on sequence peculiarities and not on functional properties.

It has been reported that acylations and hydrophobic regions present in reggie proteins are required for their membrane association [20,25]. However, it is possible that protein interactions mediated by other reggie domains might strengthen its association with cellular membranes. Accordingly, we observe that reggie-1 and -2 constructs lacking the N-terminal end (acylation sites and the first hydrophobic stretch), still significantly associate with the plasma membrane. Similarly, constructs involving only the C-terminal

“oligomerization” domains show significant association with cellular membranes. Our results suggest that the C-terminal “oligomerization” domains interact with endogenous reggie proteins at the plasma membrane. In this case, overexpression of these regions might interfere with the normal function of reggie-microdomains, acting as a dominant negative. In agreement with this view, we have recently shown that overexpression of the C-terminal “oligomerization” domain of reggie-1 severely impairs the cytoskeleton rearrangement after T cell activation [32]. Notably, membrane association of reggie-2 is stronger compared with reggie-1, probably due to the presence of a second hydrophobic stretch in the SPFH domain [33]. Indeed, a reggie-2 construct containing only the SPFH domain still associates with cellular membranes, whereas a similar construct of reggie-1, which does not possess any predicted second hydrophobic stretch, fails to associate. Recently, two putative cholesterol recognition/interaction amino acid (CRAC) motifs have been predicted to support membrane association of reggie-1 [34]. However, these motifs might not be functionally significant, because our reggie-1 construct containing only the SPFH region does not associate with cellular membranes.

Reggie-2 needs the presence of reggie-1 to be stable at the protein level

Further evidence for the interaction between reggie-1 and reggie-2 is provided by our siRNA studies: downregulation of reggie-1 causes the concomitant loss of reggie-2 protein, but not vice versa. This effect can be blocked using lactacystin, indicating that in the absence of reggie-1, reggie-2 undergoes proteasomal degradation rather than unspecific blockage of translation. Since *Drosophila* reggie-1 is also required to maintain protein stability of reggie-2 [18], it appears that this type of protein regulation in reggie-microdomains is highly conserved among metazoans. Interestingly, similar dependencies have been reported for other members of the SPFH family: prohibitin-1 null mutants in *Saccharomyces cerevisiae* lose prohibitin-2 protein and vice versa [35], and HflK and HflC appear to be interdependent as well [36], although the participation of the proteasome in these degradation processes remains elusive. It is likely that in all these cases the stability of one of the proteins is closely related to the formation of hetero-oligomers. In the case of reggies, however, this phenomenon might be more complex, since reggie-1 and -2 can localize in different sub-cellular compartments in PC-3 cells during the cell cycle [37], and in cell types such as the mouse macrophage RAW 264.7 and fibroblast 3T3 (our own unpublished results). Further studies are required to clarify this issue.

Our data indicate that reggie homo- and hetero-tetramers are the building blocks of larger multimeric clusters at the plasma membrane, in which reggie-2 requires reggie-1 for its stabilization. The ultimate size of reggie structures remains elusive, although from our early immunoelectron microscopy data -where the reggie-microdomains were calculated roughly to 0.05 to 0.1 μm [13]- we estimate that several hundred reggie proteins contribute to form such clusters. Taken together, we have uncovered the molecular

determinants for the formation and stabilization of reggie-microdomains, which will be helpful for understanding the functions of reggies as scaffolding proteins involved in signaling across the plasma membrane.

Abbreviations footnote

¹The abbreviations used are: SPFH, stomatin/prohibitin/flotillin/HflK/C; DSS, Disuccinimidyl suberate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; DMS, dimethyl suberimidate; DST, disuccinimidyl tartarate; N2a, Neuro 2a; MEM, minimal essential medium; mAb, monoclonal antibody; pAb, polyclonal antibody; HRP, horseradish peroxidase; PBS, phosphate buffered saline; EGFP, enhanced green fluorescent protein; BiFC, bimolecular fluorescence complementation; YFP, yellow fluorescent protein; NCAM, neural cell adhesion molecule; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Acknowledgements

We thank Matthias F. Langhorst for critical review of the manuscript and Kay Diederichs for the constructive discussion about structural modeling. This work was supported by grants from the Ministerium Forschung, Wissenschaft und Kunst Baden-Württemberg (TSE program), the Deutsche Forschungsgemeinschaft (SFB TR-11) and the Fonds der Chemischen Industrie.

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FIGURE LEGENDS

Figure 1 Schematic representation of reggie-1 and -2 domains and deletion constructs

The N-terminal SPFH domains are indicated in grey, the C-terminal “flotillin” domains in white, and the predicted PDZ3 domains in dark grey. Acylations occurs in the regions indicated by diagonal black stripes and the hydrophobic stretches are shown as black bars. The predicted coiled-coil (CC) stretches of reggie-1 are indicated.

Figure 2 Endogenous reggie-1 and -2 form tetramers in N2a cells after DSS-based cross-linking

N2a cells were subjected to chemical cross-linking using 1 mM of DSS over the times indicated above each lane. Reggie-1 (*left panel*) and -2 (*right panel*) specific mAbs were used for detection and reveal ~47 kDa reggie-1 and -2. Reggie tetramers migrate as ~200 kDa bands (*arrowheads*).

Figure 3 Urea treatments and homo-tetramerization of EGFP- and BiFC-reggie-1 and -2 constructs in N2a cells

A, N2a cells were lysed using the concentration of urea (M) indicated above each lane. Endogenous reggie-1 tetramers migrate as ~200 kDa bands (*arrowheads*) in a normal SDS-PAGE (*left panel*) and in a

strong denaturing 6 M urea-SDS-PAGE (*right panel*). *B*, Transiently transfected N2a cells expressing EGFP- or BiFC-fusion proteins indicated above each lane and non-transfected cells (*N2a*) were cross-linked using 1 mM of DSS for 5 min after 20 h of transfection. Reggie-1 (*left panel*) and -2 (*right panel*) specific mAbs used for detection reveal endogenous reggie monomers of ~47 kDa (*), as well as the ~60 and ~75 kDa monomers of reggie-BiFC and reggie-EGFP fusion proteins, respectively (*open and closed arrowheads, respectively*). Tetramers of endogenous reggie proteins of ~200 kDa are also evident (+). Homo-tetramers of reggie-BiFC and reggie-EGFP migrate as ~240 and ~300 kDa bands, respectively (*dark and light grey arrowheads, respectively*).

Figure 4 Homo- and hetero-tetramerization of HA-tagged and EGFP-fused reggie-1 and -2 in N2a cells

N2a cells were transiently co-transfected with the constructs indicated above each lane. Cells were cross-linked 20 h post transfection using 1 mM of DSS for 5 min. Reggie-1 (*left panel*) and -2 (*right panel*) specific mAbs revealed endogenous reggie tetramers of ~200 kDa (+). Reggie-EGFP homo-tetramers migrate as ~300 kDa (*open arrowheads*), and EGFP- and HA-containing hetero-tetramers migrate as ~250 kDa bands (*closed arrowheads*).

Figure 5 The C-terminal halves of reggie-1 and -2 are responsible for oligomerization

Transiently transfected N2a cells expressing HA-tagged reggie-1 (*A* and *C*) and reggie-2 (*B*) constructs indicated above the lanes were either cross-linked using 1 mM of DSS for 5 min (+) or left untreated (-). *A* and *B*, Samples blotted using mAb against HA-tag reveal bands indicative of oligomers of constructs including the C-terminal domain of reggie-1 (HA-R1₄₁₋₄₂₈ and HA-R1₁₈₄₋₄₂₈) and reggie-2 (HA-R2₃₆₋₄₂₈ and HA-R2₁₈₀₋₄₂₈). Constructs including only the SPFH domain of both reggies (HA-R1₄₁₋₁₈₃ and HA-R2₃₆₋₁₇₉) fail to form oligomers under this conditions, indicating that the C-termini are necessary and sufficient for oligomerization. *C*, Arrowheads show the corresponding homo-tetramers of reggie-1 species including the second predicted coiled-coil stretch (HA-R1₄₁₋₃₂₁ and HA-R1₄₁₋₃₅₃). Reggie-1 constructs lacking this stretch (HA-R1₄₁₋₂₃₈ and HA-R1_{41-238/321-353}) fail to form homo-tetramers, although some smaller oligomers are also observed.

Figure 6 Membrane localization of HA-tagged truncated reggie constructs

A-F: Overlay of confocal fluorescence images corresponding to staining of NCAM (green) and HA-tagged reggie constructs (red), as well as the corresponding distribution profiles. N2a cells were transiently transfected with vectors encoding HA-R1₄₁₋₄₂₈ (*A*), HA-R1₄₁₋₁₈₃ (*B*), HA-R1₁₈₄₋₄₂₈ (*C*), HA-R2₃₆₋₄₂₈ (*D*), HA-R2₃₆₋₁₇₉ (*E*), and HA-R2₁₈₀₋₄₂₈ (*F*) 20 h prior to analysis using Abs specific against NCAM and the HA tag. Note that reggie-1 constructs including the C-terminal halves (HA-R1₄₁₋₄₂₈ and HA-R1₁₈₄₋₄₂₈)

colocalize with NCAM, while a construct including the SPFH domain only (HA-R1₄₁₋₁₈₃) fails to colocalize with NCAM at the plasma membrane as indicated in the profile graphic. All reggie-2 constructs tested (HA-R2₃₆₋₄₂₈, HA-R2₃₆₋₁₇₉ and HA-R2₁₈₀₋₄₂₈) differentially colocalize with NCAM at the plasma membrane.

Figure 7 Cell fractionation of N2a cells expressing HA-reggie constructs

Transiently transfected N2a cells expressing HA-tagged reggie-1 (*A* and *C*) and reggie-2 (*B*) constructs indicated above the lanes were fractionated by high-speed centrifugation. Aliquots of the fractions corresponding to the whole cell extracts (*Ext*), cytosol (*Cyto*) and membrane (*Mem*) were analyzed by Western blot using a mAb against HA-tag. *A*, Reggie-1 constructs including the C-terminal halves (HA-R1₄₁₋₄₂₈ and HA-R1₁₈₄₋₄₂₈) are present in both cytosolic and the membrane fractions, whereas a construct including the SPFH domain only (HA-R1₄₁₋₁₈₃) is not significantly present in the membrane fraction. *B*, All the reggie-2 constructs tested (HA-R2₃₆₋₄₂₈, HA-R2₃₆₋₁₇₉ and HA-R2₁₈₀₋₄₂₈) are significantly present in both cytosolic and membrane fractions. Some small molecular weight bands are observed for HA-R1₁₈₄₋₄₂₈ and HA-R2₁₈₀₋₄₂₈ constructs, which might represent degradation products after the fractionation process. *C*, The reggie-1 construct including the first predicted coiled-coil stretch (HA-R1₄₁₋₂₃₈) is significantly present in both cytosolic and membrane fractions, whereas the control construct (HA-R1₄₁₋₁₈₃) is largely cytosolic.

Figure 8 siRNA mediated knockdown of reggie-1 induces proteasomal degradation of the reggie-2 protein

N2a cells were transfected with two different siRNAs against reggie-1 (R1.0 and R1.1), one siRNA against reggie-2 (R2.0) and a non-specific control siRNA against firefly luciferase (GL2). Additional control cells treated with the transfection reagent Lipofectamine 2000 alone were examined (Control). *A*, RT-PCR analyses of total mRNA at 48 h post transfection show that reggie-1 and -2 mRNAs are specifically downregulated by the corresponding siRNAs. GAPDH was used as control for the amount of cDNA. *B*, Protein analyses of siRNA mediated knockdown by Western blotting show that both siRNAs against reggie-1 (R1.0 and R1.1) cause depletion of reggie-1 as well as reggie-2 protein, while siRNA against reggie-2 (R2.0) only downregulates reggie-2 protein. Incubation with 10 μ M of lactacystin blocks this effect, indicating that reggie-2 undergoes proteasomal degradation in the absence of reggie-1. Specific mAb against GAPDH was used as loading control.

Figure 1

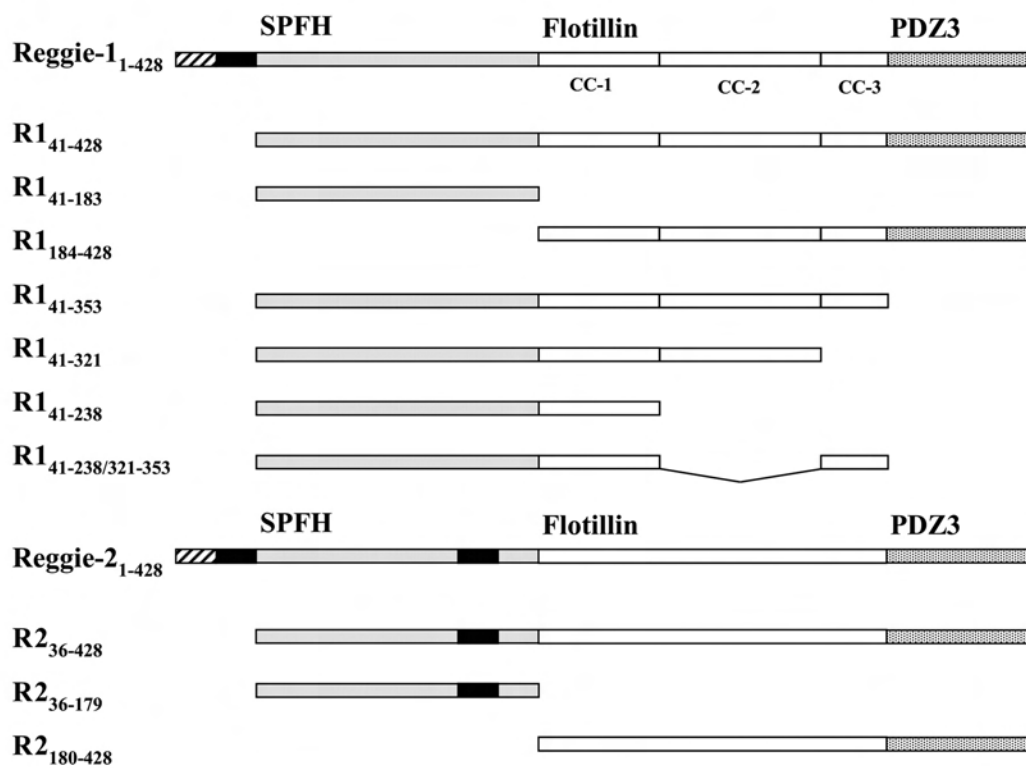


Figure 2

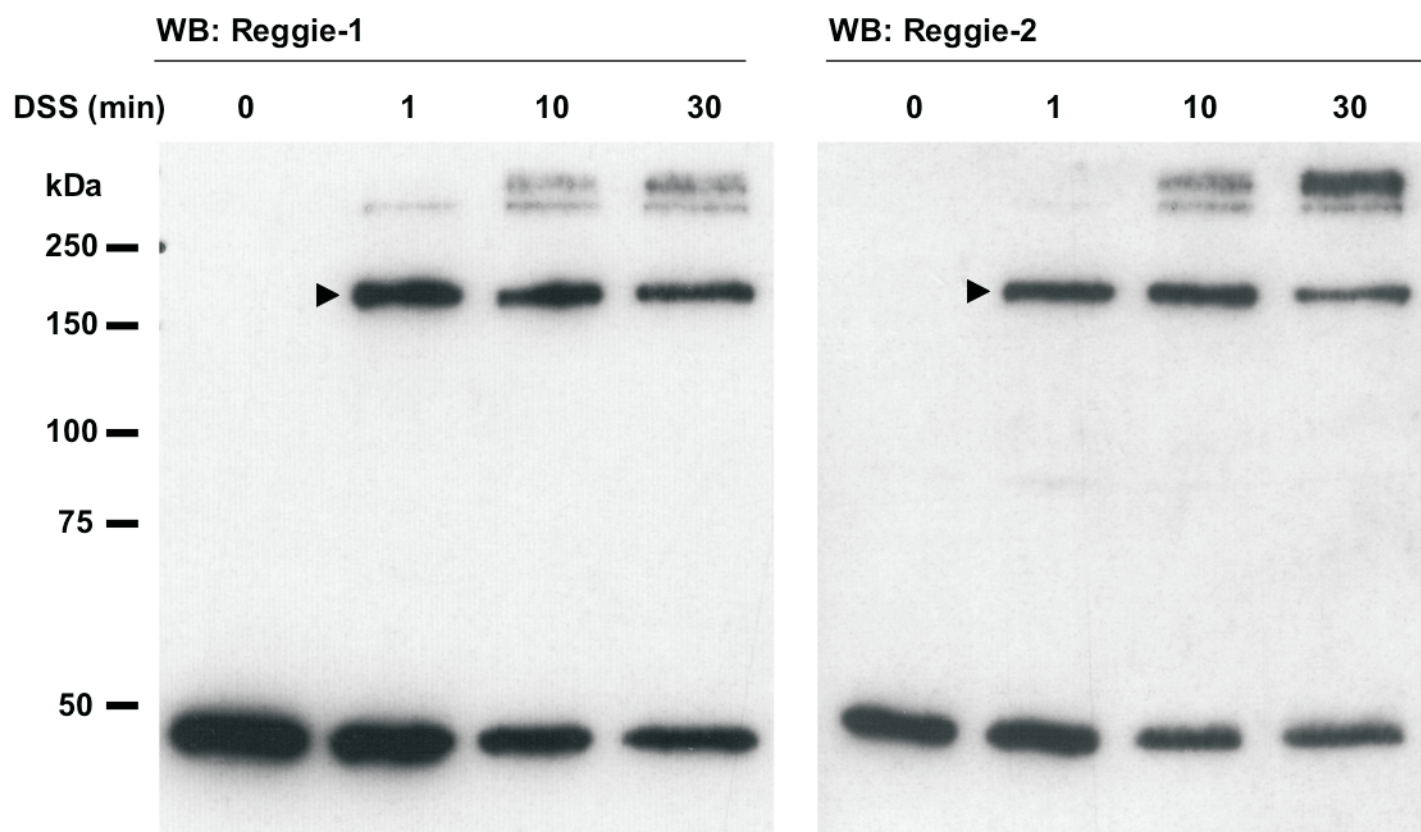


Figure 3

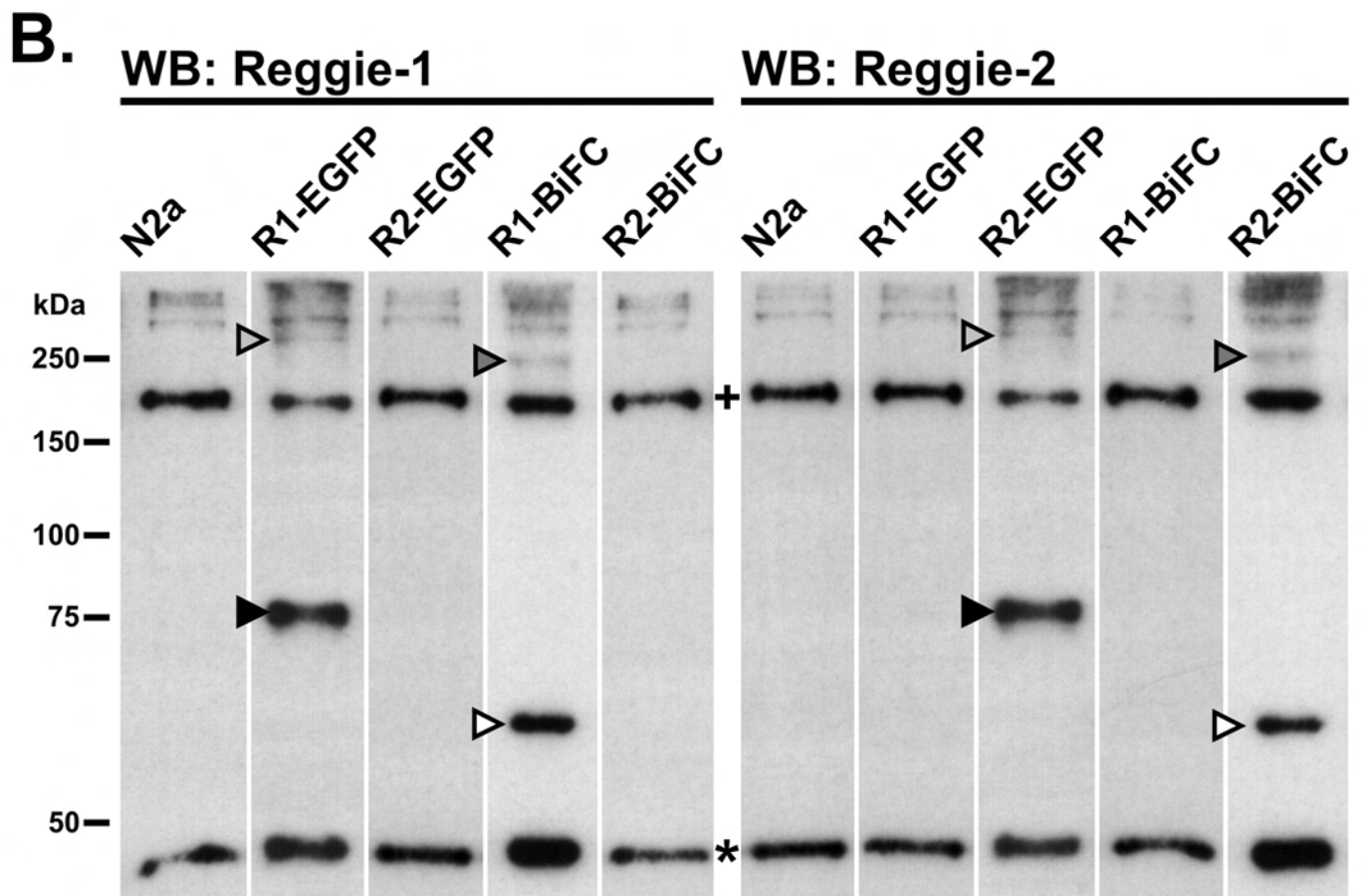
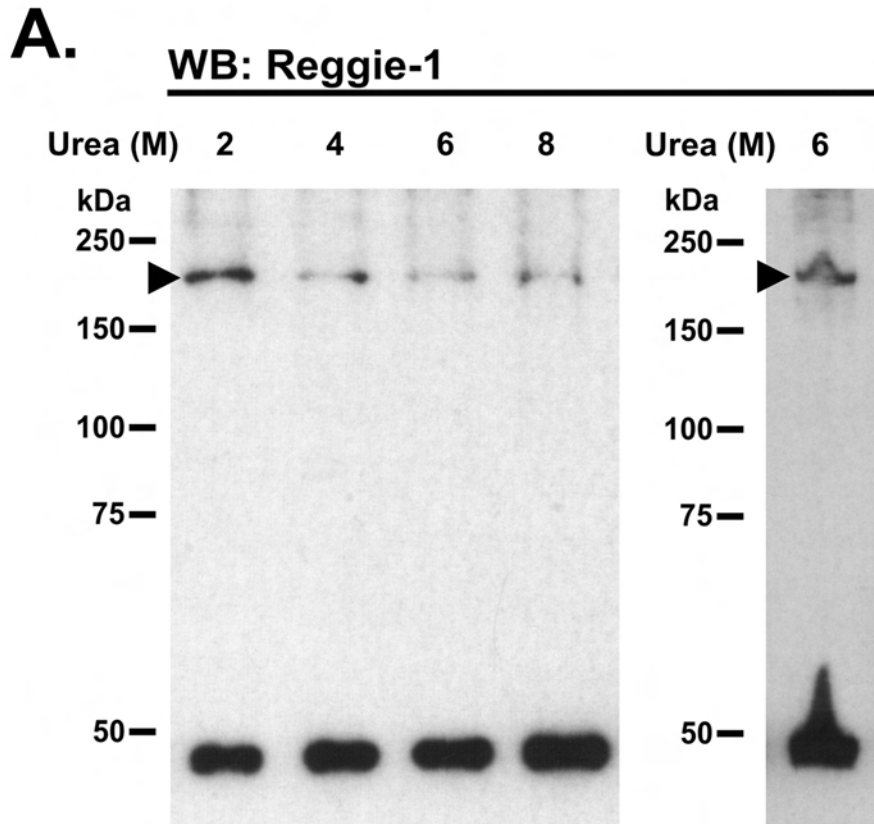


Figure 4

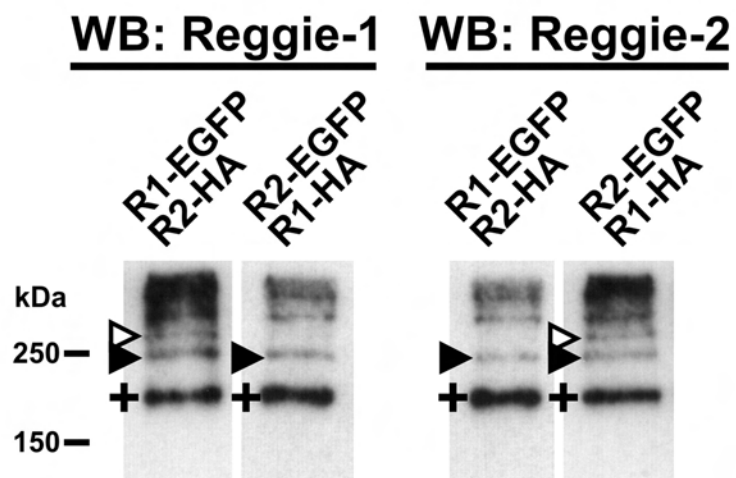


Figure 5

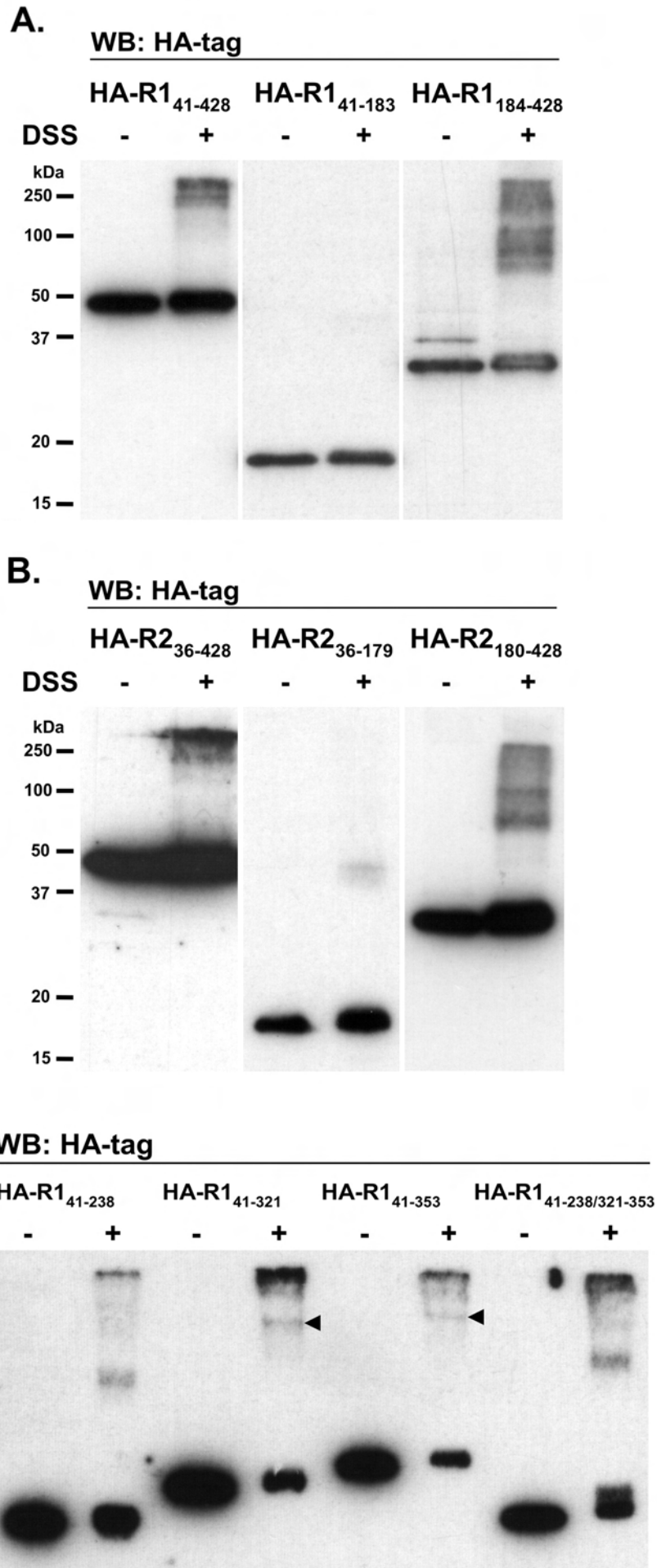


Figure 6

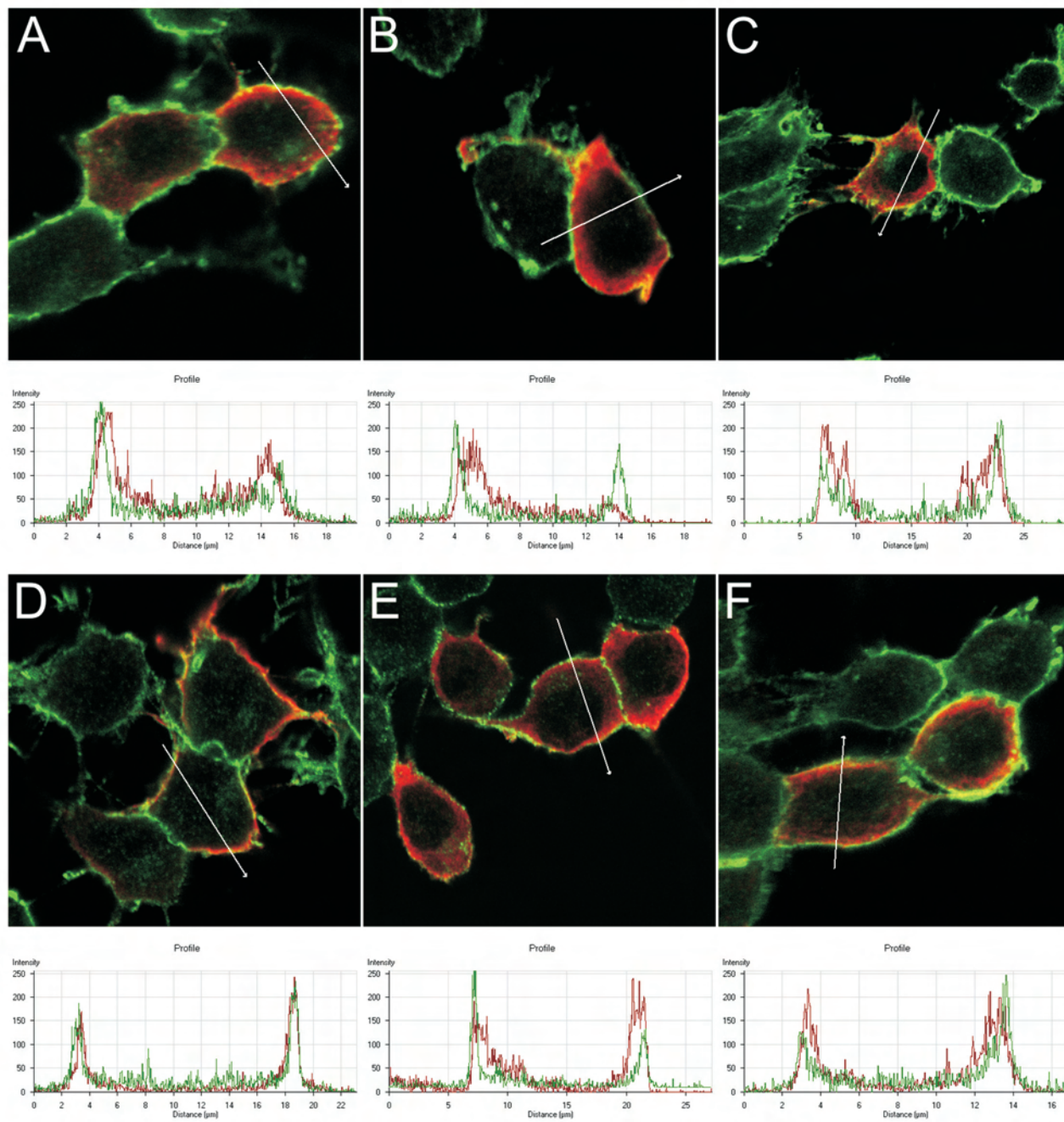
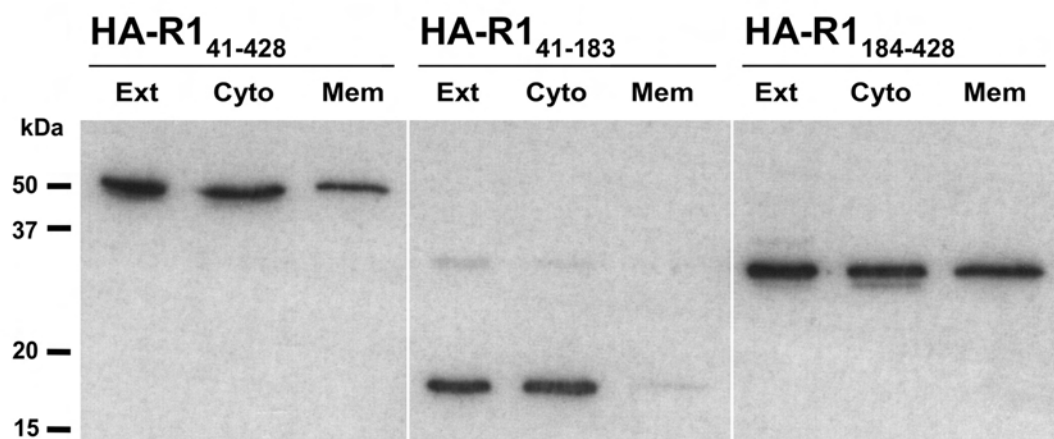
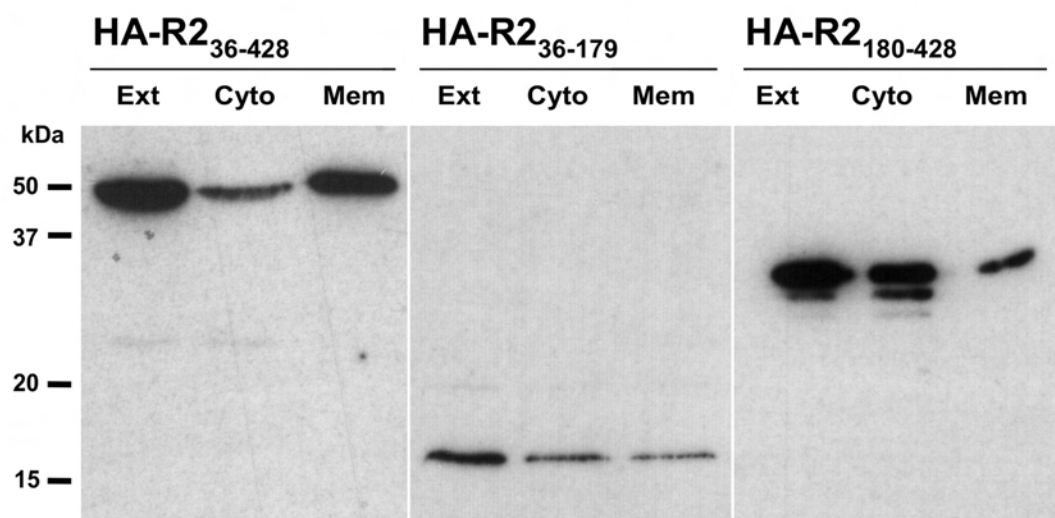


Figure 7

A. WB: HA-tag



B. WB: HA-tag



C. WB: HA-tag

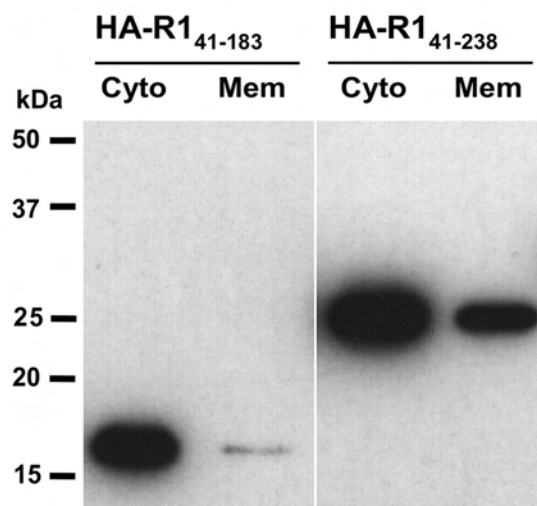


Figure 8

