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Functional and physical interactions between formyl-peptide-receptors and scavenger receptor MARCO and their involvement in amyloid beta 1–42-induced signal transduction in glial cells

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Abstract

Recent studies suggest that the chemotactic G protein-coupled receptor formyl-peptide-receptor-like-1 (FPRL1) or the scavenger receptor MARCO (macrophage receptor with collagenous structure) plays an essential role in the inflammatory response of host defense mechanisms and neurodegenerative disorders such as Alzheimer's disease. We therefore analyzed the involvement of FPRL1 and MARCO in amyloid β1–42 (Aβ1–42)-induced signalling by extracellular-signal regulated kinases 1/2 (ERK1/2) phosphorylation and cAMP level measurement in glial cells (astrocytes and microglia) and in transfected HEK293 cells. Receptors were inhibited by small interference RNA and the consequences in Aβ1–42- and MARCO agonist fucoidan-induced signal transduction were determined. Receptor deactivation by antagonists or small interference RNA verified the importance of FPRL1 for Aβ1–42-mediated signal transduction by ERK1/2 phosphorylation and cAMP level measurement in glial cells. Furthermore, for the first time, we have demonstrated a functional interaction between FPRL1 and scavenger receptors in fucoidan-mediated signalling by ERK1/2 phosphorylation and cAMP level measurement. In addition, co-immunoprecipitation data and fluorescence microscopy measurements revealed a physical interaction between FPR, FPRL1 and MARCO. These results suggest that FPRL1 plays a pivotal role for Aβ1–42-induced signal transduction in glial cells and the interaction with MARCO could explain the broad ligand spectrum of formyl peptide receptors.

Keywords: amyloid beta 1–42, formyl peptide receptor, glial cell, MARCO, signal transduction.

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Alzheimer's disease is a neurodegenerative disorder characterized by senile plaques and neurofibrillary tangles (Selkoe 2001). An excessive activation of glial cells (astrocytes and microglia) prior to neuronal death is likely to be caused initially by the β-amyloid peptide (Aβ1–42) in the brain (a 42 amino acid form), which plays a central role in mediating neurotoxicity and the formation of senile plaques. Cytokines and chemokines are released by activated glial cells via elevated levels of both non-fibrillar (Lambert *et al.* 1998; Hartley *et al.* 1999) and fibrillar Aβ1–42 (Lorenzo and Yankner 1994). This may subsequently lead to gliosis and cytotoxicity in neurons (Eikelenboom *et al.* 2002). In addition, glial cells may damage neurons by releasing reactive oxygen species (Qin *et al.* 2002; Abramov *et al.* 2004). However, the role of glial cells in the formation of amyloid plaques in Alzheimer's disease remains unknown (Nagele *et al.* 2002, 2004). The underlying pathogenic mechanisms are not well understood, especially regarding the initial steps of cellular Aβ1–42 uptake and induction of

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Abbreviations used: Aβ, β-amyloid peptide; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinases; FCS, fetal calf serum; fMLF, formyl-methionyl-leucyl-proline; FPRL1, formyl-peptide-receptor-like 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MARCO, macrophage receptor with collagenous structure; RAGE, receptor for advanced glycation endproducts; SDS, sodium dodecyl sulphate; siRNA, small interference RNA.

signal transduction. Recent studies suggest that the G protein-coupled receptor formyl-peptide-receptor-like 1 (FPRL1) is involved in Aβ1–42- and $PrP_{106–126}$-induced activation and internalization in glial cells (Brandenburg *et al.* 2007, 2008).

However, one should also take note of further receptors. In fact, the putative role of the scavenger receptor MARCO (macrophage receptor with collagenous structure) has been discussed for internalization and Aβ1–42-mediated microglia activation (Paresce *et al.* 1996; Bamberger *et al.* 2003; Koenigsknecht and Landreth 2004). MARCO is a membrane glycoprotein that can bind chemically modified low-density lipoprotein or Gram-positive and Gram negative bacteria (Kraal *et al.* 2000; Mukhopadhyay and Gordon 2004). Alarcon *et al.* (2005) was able to show the expression of MARCO in glial cells. Moreover, the advanced glycation end-product-specific receptor, receptor for advanced glycation endproducts (RAGE), which is expressed in neurons and microglia cells, in which it binds Aβ1–42 with high affinity (Paresce *et al.* 1996; Yan *et al.* 1996; El Khoury *et al.* 1998). As some studies have failed to confirm the ability of MARCO or RAGE to mediate the proinflammatory activity of Aβ1–42 in mononuclear phagocytes further studies must clarify such interaction (McDonald *et al.* 1998; Combs *et al.* 1999; Antic *et al.* 2000; Lorton *et al.* 2000).

This article describes our investigation of the involvement of FPRL1 and MARCO in Aβ1–42-induced signalling, in which we measured extracellular-signal regulated kinases 1/2 (ERK1/2) phosphorylation and cAMP levels in glial cells and transfected HEK293 cells. The importance of FPRL1 for Aβ1–42-mediated signals transduction in glial cells was verified by antagonist- or small interference RNA (siRNA)-induced receptor inactivation. For the first time, this study shows functional und physical interaction between FPR, FPRL1 and MARCO using co-immunoprecipitation and fluorescence microscopy. Furthermore, this study demonstrates the involvement of FPRL1 in MARCO-mediated signalling for fucoidan treatment. Fucoidan is an agonist for the scavenger receptor MARCO (Bamberger *et al.* 2003). The results suggest that FPRL1 plays a pivotal role in Aβ1–42-induced signal transduction in glial cells, and also show the capability of FPRL1 to expand its inflammatory ligands spectrum by interaction with the scavenger receptor MARCO.

Experimental procedure

Reagents

Human Aβ1–42 and the FPRL1 antagonist WRW4 were purchased from Dr. P. Henklein (Charité, Berlin, Germany). Peptides were dissolved at 1 mM and 10 mM concentration in dimethylsulfoxide, respectively and Aβ1–42 is present in soluble form. dimethylsulfoxide, used as vehicle in a concentration of 0.1%, showed no significant effects in the experiments. Fucoidan and formyl-methionyl-leucyl-proline (fMLF) were purchased from Sigma (Deisenhofen, Germany) and dissolved in Aqua bidest.

Cloning of cDNA and plasmid

The human MARCO (hMARCO; NM_006770) was subcloned into the pEAK10 expression vector containing a puromycin resistance gene (Edge Bio Systems, Gaithersburg, MD, USA). The process yielded plasmid pEAK10-hMARCO. The pcDNA3.1-hFPRL1 plasmid containing a neomycin resistance gene was kindly provided by Dr. U. Rescher (Münster, Germany).

Cell culture

Using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol, HEK293 cells (American Type Culture Collection, Rockville, MD, USA) were first transfected with either pEAK10-hMARCO or pcDNA3.1-hFPRL1 plasmid. Stable transfectants were selected in the presence of 1 μg/mL puromycin (Sigma) or 500 μg/mL G418 (Carl Roth, Karlsruhe, Germany). To generate hFPRL1 cell lines co-expressing hMARCO, cells were subjected to a second round of transfection with pEAK10-hMARCO and selected in the presence of 1 μg/mL puromycin and 500 μg/mL G418.

Isolated cerebral cortices and rostral mesencephali from 2-day-old rats were stripped of the meninges, minced and dissociated enzymatically with trypsin from bovine pancreas (Sigma) in phosphate-buffered saline and 50 μg/mL DNAseI (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min at 37°C and crushed mechanically with Pasteur pipettes. Astrocytes were prepared following the McCarthy and DeVellis method (McCarthy and de Vellis 1980), which allows for the preparation of nearly pure cultures of astrocytes (> 97%) and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Suspended microglial cells were plated in 75 cm^2 cell culture flasks in microglial cell growth medium and harvested as described (Wilms *et al.* 2003). Prior to replating microglial cells for different assays, cell number and viability was estimated by trypan-blue exclusion. This procedure increased the purity of the microglial preparation to > 98% with few astrocytes remaining. To test cell purity, cultures were stained with specific cell markers for astrocytes (glial fibrillary acidic protein (GFAP); astrocytes marker; Sigma) and microglia (OX42; microglia/macrophages marker; Sera-Lab, Leicestershire, UK).

Astrocytes siRNA transfection

One day before transfection, 3×10^5/well astrocytes were seeded in DMEM containing 10% FCS in 6-well-plates and transfected with Primefect® (Lonza, Riverside, USA) transfecting agent containing control siRNA (25 nM) and siRNA for target proteins (25 nM), respectively according to the manufacturer's recommendation. Small interfering RNA (siRNA) duplexes corresponding to rat FPR, FPRL1 and MARCO cDNA sequences (GenBank accession number XM_001057934, XM_218012 and XM_001054109) or control siRNA (target sequence: 5′-AATTCTCCGAAGGTGTCACGT-3′) were purchased from Qiagen, Valencia, CA, USA. The rat FPR, FPRL1 and MARCO sequences chosen for performing RNA interference targeting were 5′-AAGTTCATATATACTGTAATA-3′, 5′-AACCAGTGATACAGGCACAAA-3′ and 5′-AAGGGAGAACCTGGGATGAAA-3′. Cells were cultured for an additional 96 h and

analyzed for mRNA and protein expression via SYBR green real time PCR and western blotting, respectively.

Western blotting and immunoprecipitation

For western blot analysis of FPR, FPRL1 and MARCO, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and MAPKs phosphorylation, astrocytes, microglia or transfected HEK293 cells were seeded in DMEM containing 10% FCS. Cells were harvested in a lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton, 2 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM glycerol 2-phosphate, 1 mM phenylmethylsulfonylfluoride), and protein aliquots (30 μg for FPR, FPRL1 and MARCO; 3 μg for GAPDH and 5 μg for pERK and ERK2) were resolved in sodium dodecyl sulphate (SDS) sample buffer for SDS–polyacrylamide gel electrophoresis. Western blotting was conducted, and resulting polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Essex, UK) were incubated with polyclonal primary antibodies against FPR (1 : 100), FPRL1 (1 : 100), GAPDH (1 : 250), pERK1/2 (1 : 500), ERK2 (1 : 500; all from Santa Cruz, Santa Cruz, CA, USA) and MARCO (1 : 1000; Serotec, Oxford, UK) and dissolved in Tris-buffered saline-T [20 mM Tris, 0.14 M NaCl, 1.00 mM EDTA, 0.1% (v/v) Tween 20, adjusted to pH 7.5 with HCl] overnight at 4°C. Peroxidase-labeled secondary antibodies were used for subsequent detection by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Cells (1.5×10^6/plates for astrocytes and transfected HEK293 cells, 10×10^6/plates for microglia) were plated onto 100-mm dishes and grown to 80% confluence. When indicated, cells were exposed to 1 μM Aβ1–42 and 100 μg/mL fucoidan for 30 min. Cells were washed twice with phosphate-buffered saline and harvested into ice-cold lysis buffer (10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 10 μM iodoacetamide, and a mixture of proteinase inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, and 10 μg/mL bacitracin). Subsequently, the cell suspensions were incubated for 30 min on ice and homogenized, and the homogenates were centrifuged at 500 *g* for 5 min at 4°C to remove non- unlocked cells and nuclei. Membranes were then pelleted at 20 000 *g* for 30 min at 4°C, and pellets were lysed in detergent buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 3 mM EGTA, 4 mg/mL β-dodecylmaltoside, and the proteinase inhibitors listed above) for 1 h on ice. Lysates were centrifuged at 20 000 *g* for 30 min at 4°C, and the protein content of the resulting supernatant was determined using a BCA protein assay (Pierce, Rockford, IL, USA). Receptor proteins were immunoprecipitated with 50 μL protein G agarose beads preloaded with 5 μg anti-FPR or anti-FPRL1 antibodies (for rat glial cells, from Santa Cruz; for HEK cells transfected with hFPRL1, from MBL, Woburn, MA, USA) overnight at 4°C. Beads were washed five times with detergent buffer and eluted into 200 μL of SDS-sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 100 mM DL-dithiotreitol, and 0.005% bromphenol blue) at 60°C for 20 min. After SDS–polyacrylamide gel electrophoresis and electroblotting, membranes were incubated with rabbit anti-FPR, FPRL1, MARCO (for rat glial cells, from Serotec; for transfected HEK cells, from Hycult Biotech, Uden Netherland) or phosphoserine/-threonine/-tyrosine (1 : 500; GeneTex, Irvine, CA USA) antibodies overnight at 4°C. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system mentioned above.

Determination of receptor activity by measurement of cAMP accumulation

1.5×10^5 astrocytes/well, or transfected HEK293 cells or 5×10^5 microglia/well were seeded in 22-mm 12-well dishes with DMEM containing 10% FCS and incubated overnight. The medium was removed and replaced by 0.5 mL of serum-free DMEM medium containing 10 μM forskolin (for astrocytes; Sigma) or 25 μM forskolin (for microglia or HEK293 cells) plus agonists. Different forskolin concentrations were used because of different cell sensitivities to forskolin-stimulated adenylate cyclase activity. The cells were incubated at 37°C for 15 min, and the reaction was terminated by removal of the culture medium and addition of 1 mL of ice-cold HCl/ethanol (1 N; 1 : 100, v/v). After centrifugation the supernatant was evaporated, residues were dissolved in Tris-EDTA (TE) buffer (50 mM Tris–EDTA, pH 7.5), and cAMP content was determined using a commercially available radioimmunoassay kit (Amersham Pharmacia Biotech).

Fluorescence microscopy

Glial cells were grown on glass coverslips. Cells were then fixed with 4% paraformaldehyde and 0.2% picric acid in a phosphate buffer at pH 6.9 (Zamboni and De Martino 1967) for 30 min, and then permeabilized with ice-cold methanol for 10 min. Subsequently they were incubated with polyclonal goat anti-FPR (1 : 100), rabbit anti-FPRL1 (1 : 100) and mouse anti-MARCO (1 : 250) antibodies for 1 h at 20°C in order to detect an extracellular domain of the rat (or mouse and human) FPRL1 receptor. Finally, the cells were incubated with rabbit anti-goat or sheep anti-rabbit Cy3 (Sigma) and goat anti-mouse AlexaFluor 488 (Molecular Probes, Eugene, OR, USA). Bisbenzimide (Sigma) was used for nuclear staining and cells were photographed using a Zeiss Axiovert microscope (Zeiss, Göttingen, Germany). Cross-reactivity between FPRL1 and FPR was not observed in the immunofluorescence stainings or in the western blot analysis using the anti-FPRL1 antibodies (data not shown).

RNA isolation, RT-PCR and real time RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were reverse-transcribed by moloney murine leukemia virus reverse transcriptase (Superscript RT; Gibco, Rockville, MD, USA) and oligo-$(dT)_{15}$ primers (Promega, Madison, WI, USA). The cDNA products were immediately used for SYBR green real time RT-PCR and gene expression of FPR, FPRL1 and MARCO was monitored using an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) according to the standard procedure. The RT-PCR or real time RT-PCR cycles included 40 cycles of general denaturation at 94°C (30 s), annealing and elongation at 60°C (45 s), except for the first cycle which had a 15 min denaturation and the last cycle which had a 7 min elongation at 72°C. The primer for FPR, FPRL1 and MARCO were manufactured by Qiagen (QuantiTect Primer Assay), and the specificity of the amplification reaction was determined by melting curve analysis. Signals of the different genes were normalized against those of glycerinaldehyde-3-phosphate dehydrogenase (GAPDH forward primer: 5′-TCTACCCACGGCAAGTTCAAC-3′; reverse

primer: 5′-TCTCGCTCCTGGAAGATGGT-3′) in order to obtained quantitative results by SYBR green real time RT-PCR. Primer sequences for RT-PCR, PCR conditions and the expected molecular masses of PCR products are summarized in supporting Table S1. The PCR products were electrophorized on a 2% agarose gel and visualized with ethidium bromide incorporation under UV light.

Statistical analysis

All experiments were performed in triplicate and figures are shown for representative experiments. The significance of the difference between experimental and control groups was analyzed using ANOVA followed by the Bonferroni test. Data from SYBR green real time RT-PCR, from densitometric quantification of western blots and from cAMP assays were analyzed by GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Inhibition of Aβ1–42- and fucoidan-induced ERK1/2 phosphorylation and change of cAMP levels by FPRL1 antagonist WRW4 in glial cells

To investigate both the effect of Aβ1–42 and MARCO and that of fucoidan on glial cell activation, we incubated primary rat astrocytes and microglia with Aβ1–42 and fucoidan to determine ERK1/2 phosphorylation and the inhibition of forskolin-induced cAMP accumulation. As shown in Fig. 1(a–d), treatment with Aβ1–42 and fucoidan resulted in an intense phosphorylation of ERK1/2. As shown very recently, the FPRL1 antagonist WRW4 inhibits the phosphorylation after stimulation with Aβ1–42 (Brandenburg *et al.* 2008). Here we show that the phosphorylation of ERK1/2 by fucoidan is also inhibited by WRW4, which has no effect on ERK1/2 phosphorylation by itself. To test whether Aβ1–42 and fucoidan are competing for the same signal transduction pathway, we incubated the glial cells with Aβ1–42 and fucoidan simultaneously. Without fucoidan pre-incubation, the phosphorylation of ERK1/2 does not differ from that under stimulation with Aβ1–42 and fucoidan alone. Pre-incubation with fucoidan for about 15 min slightly inhibited ERK1/2 phosphorylation, whereas phosphorylation was completely blocked using 30 min pre-incubation time. No changes could be detected using the control peptide Aβ42–1, as described recently (Brandenburg *et al.* 2008). In addition to the assay mentioned above, the FPRL1 receptor capacity to inhibit cAMP formation was investigated. The FPRL1 is coupled to inhibitory G-protein (G_i), and the activation resulted in a reduction of cAMP level. Forskolin was used as the activator of the adenylate cyclase (Brandenburg *et al.* 2007). To determine whether Aβ1–42 and fucoidan-induced cAMP formation are linked via G_i receptor activity, the cAMP production in glial cells was induced by forskolin treatment, and the interference of Aβ1–42 and fucoidan was analyzed. As shown in Fig. 1(e) and (f), the treatment with forskolin resulted in a fivefold increase of intracellular cAMP for astrocytes and microglia (up to 5 pmol) as compared to untreated cells. Application of both Aβ1–42 and fucoidan counteracted forskolin-induced cAMP formation in glial cells by approximately 80%. Figure 1(e) and (f) show that the Aβ1–42-mediated decrease of cAMP level was strongly inhibited by the FPRL1 antagonist WRW4. Moreover, the fucoidan-induced decrease of cAMP level was also blocked by the FPRL1 antagonist WRW4. These results show that Aβ1–42 is an agonist for the G protein-coupled receptor FPRL1 and that FPRL1 is involved in scavenger receptor/MARCO signalling. To test whether fucoidan pre-incubation inhibited Aβ1–42-induced decrease of cAMP level, we incubated Aβ1–42 and fucoidan simultaneously. Without fucoidan pre-incubation, the decrease of cAMP was not different from the stimulation with Aβ1–42 or fucoidan alone. A 15-min pre-incubation with fucoidan inhibited the decrease of cAMP slightly, whereas the change of cAMP level was completely blocked and reached forskolin-induced control cAMP level after 30 min of pre-incubation. WRW4 and the control peptide Aβ42–1 (Brandenburg *et al.* 2008) alone did not alter the forskolin-stimulated adenylate cyclase activity and did not influence forskolin-induced cAMP elevation in glial cells.

MARCO interacts with FPR and FPRL1 in glial cells

As described above, fucoidan-induced phosphorylation of ERK1/2 or reduction of cAMP level were inhibited by the FPRL1 antagonist WRW4. One can therefore assume that the effects of MARCO are partly mediated by FPRL1. To check this hypothesis, co-immunoprecipitation studies were conducted using formyl peptide receptor FPR or FPRL1 and MARCO or phosphoserine/-threonine/-tyrosine antibodies. FPR or FPRL1 receptors were precipitated from lysates of glial cells using anti-FPR or FPRL1 antibodies. The precipitates were immunoblotted with antibodies directed against FPR, FPRL1, MARCO or phosphoserine/-threonine/-tyrosine. As shown in Fig. 2(a), MARCO was detected in immunoprecipitates from astrocytes and microglia, suggesting that FPR or FPRL1 is physically associated with MARCO *in vitro* and/or under the experimental conditions we describe. Furthermore, neither FPR nor FPRL1 co-immunoprecipitated, which indicates that FPR is not associated with FPRL1 in glial cells *in vitro*. In summary, these results suggest that FPR or FPRL1 is physically associated with MARCO in glial cells *in vivo*. To investigate whether Aβ1–42 or fucoidan influence the amount of co-immunoprecipitated MARCO, we incubated glial cells with Aβ1–42 or fucoidan for 30 min and determined MARCO as described above. The band densities of the western blots were then evaluated by densitometric quantification. As shown in Fig. 2(b) (FPR precipitates), we did not detect an increase in co-immunoprecipitated MARCO in astrocytes or microglia in resulting FPR precipitates, whereas in the

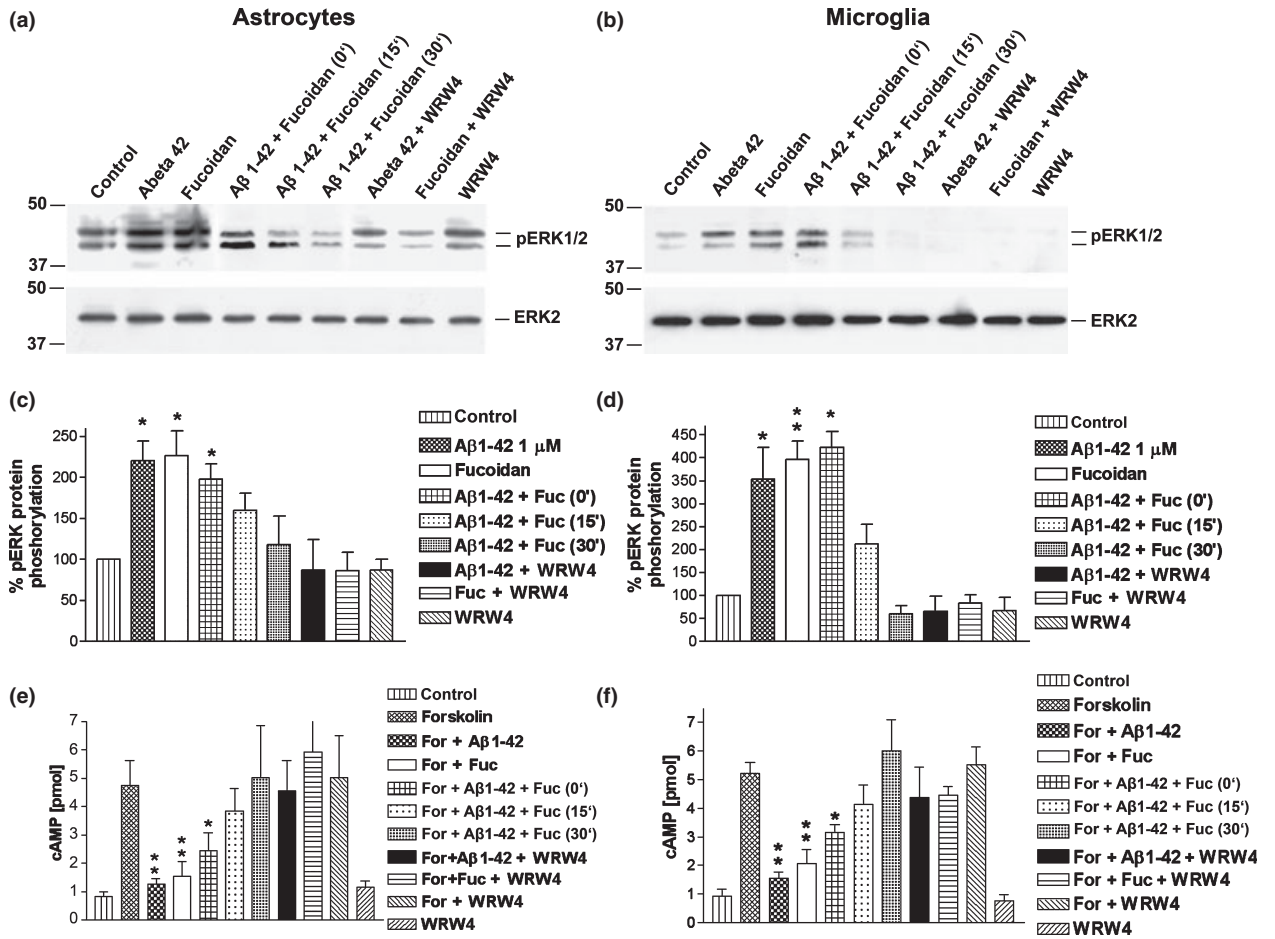

Fig. 1 Inhibition of fucoidan-induced G-protein receptor activity by the FPRL1 antagonist WRW4 in glial cells. For analysis of ERK1/2 phosphorylation, astrocytes (a) and microglia (b) were each treated with 1 μM Aβ1–42 or 100 μg/mL fucoidan with or without 10 μM WRW4 and with WRW4 alone for 5 min at 37°C. For simultaneous Aβ1–42 and fucoidan treatment, cells were pre-incubated with fucoidan for 0, 15 and 30 min and also stimulated for 5 min at 37°C. Cells were lysed, equal amounts of protein (5 μg) were dissolved in SDS sample buffer, and the levels of total ERK2 and phosphorylated ERK1/2 were determined via immunoblotting. The positions of phospho-ERK1/2 (pERK1/2) and total ERK2 (ERK2) and of the molecular mass markers (in kDa) are indicated on the right and left side, respectively. The values representing mean ± SD of phosphorylation levels derive from densitometric quantification of three independent experiments in (c) astrocytes and (d) microglia. An asterisk indicates a significant difference (*$p < 0.05$; **$p < 0.001$) compared to controls using one-way ANOVA and Bonferroni *post-hoc* tests. For analysis of inhibition of forskolin-stimulated adenylate cyclase activity, astrocytes (e) and microglia (f) were subjected to either 10 μM (e) or 25 μM (f) forskolin as well as to 1 μM Aβ1–42 or 100 μg/mL fucoidan with or without 10 μM WRW4 and to WRW4 alone for 15 min at 37°C. For simultaneous treatment with Aβ1–42 and fucoidan, cells were pre-incubated with fucoidan for 0, 15 and 30 min. cAMP levels were determined as described above (see Experimental Procedure). Values represent mean ± SD from four independent experiments. Asterisks indicate a significant difference (*$p < 0.05$; **$p < 0.001$) between forskolin plus agonists and forskolin alone, as determined by one-way ANOVA and Bonferroni *post-hoc* tests.

FPRL1 precipitates, the treatment with Aβ1–42 or fucoidan induced an increase in the amount of co-immunoprecipitated MARCO in glial cells (Fig. 2c). We also determined the agonist-induced phosphorylation of the precipitated FPR and FPRL1 after stimulation with Aβ1–42 or fucoidan. The immunoblotting with antibodies against phosphoserine/-threonine/-tyrosine shows, for FPR, no increase in receptor phosphorylation after stimulation in astrocytes and microglia (Fig. 2d). For FPRL1 precipitates the stimulation with Aβ1–42 resulted in a significant increase in receptor phosphorylation in glial cells. Interestingly, stimulation with the MARCO agonist fucoidan also increased the phosphorylation of FPRL1 in both astrocytes and microglia (Fig. 2d).

MARCO and FPR or FPRL1 co-localization in glial cells by immunofluorescence

To further substantiate our findings, we examined the distribution of FPR, FPRL1 and MARCO in glial cells using double fluorescence microscopy with receptor-specific antibodies. In astrocytes (Fig. 3a), immunofluorescent FPR

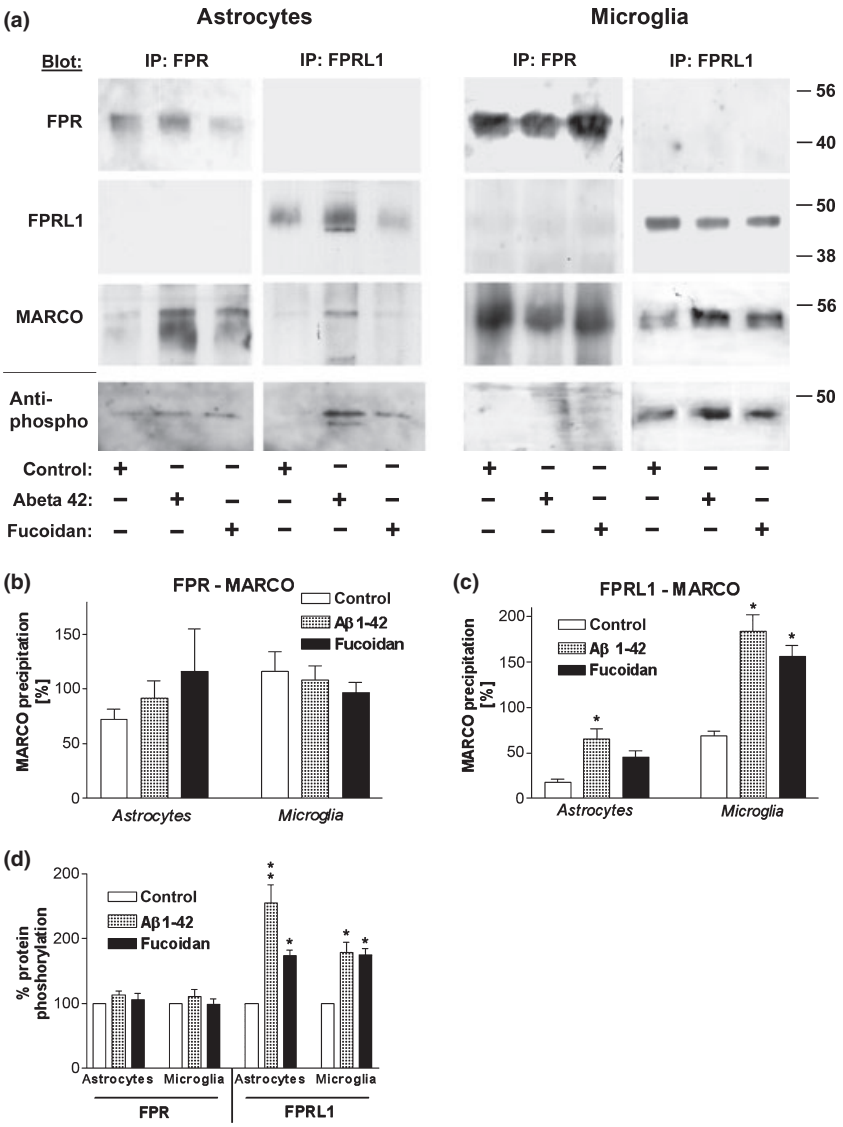

Fig. 2 MARCO interacts with FPR and FPRL1 in glial cells. Membrane proteins from astrocytes or microglia with or without 1 μM Aβ1–42 or 100 μg/mL fucoidan pre-incubation (30 min) were extracted using anti-ratFPR or ratFPRL1 antibodies. (a) The resulting immunoprecipitates were electrophoretically separated, transferred to nitrocellulose and detected with anti-FPR, anti-FPRL1, anti-MARCO and phosphoserine/threonine/tyrosine antibodies. FPR and FPRL1 are co-immunoprecipitate with MARCO in astrocytes as well as microglia. The positions of molecular mass markers are indicated on the right (in kDa). The values representing mean ± SD of protein and phosphorylation levels derive from densitometric quantification of three independent experiments for co-immunoprecipitated MARCO (b and c) and for receptor phosphorylation normalized to FPR or FPRL1 (d). An asterisk indicates a significant difference (*$p < 0.05$; **$p < 0.001$) compared to controls on the basis of one-way ANOVA and Bonferroni *post-hoc* tests.

or FPRL1 and MARCO were co-localized in both the internal compartment as well as in the plasma membrane. There was no co-localization between FPR and FPRL1. In microglia (Fig. 3b), FPR or FPRL1 showed overlapping staining with MARCO in the plasma membrane. Furthermore, co-localization between FPR and FPRL1 was detected in these cells.

Inhibition of FPR, FPRL1 or MARCO expression in astrocytes by siRNA

To determine whether FPR, FPRL1 or MARCO is essential for signal transduction of Aβ1–42, we prepared siRNA targeting rat FPR, FPRL1 and MARCO in astrocytes. Transfection of astrocytes with FPR, FPRL1 and MARCO siRNA, but not with control siRNA, resulted in a significant reduction in FPR, FPRL1 and MARCO mRNA levels 96 h after transfection as determined by SYBR green real time RT-PCR (Fig. 4a). GAPDH was used as a control. In addition, 96 h after transfection with siRNA, protein levels of FPR, FPRL1 and MARCO, as detected by western blot and evaluated by densitometric quantification, decreased in astrocytes (Fig. 4b). Protein expression was reduced by about 64%, 73% and 62% for FPR, FPRL1 and MARCO, respectively (Fig. 4c). The transfection of primary rat microglia did not reach sufficient efficiency (data not shown).

Inhibition of Aβ1–42- and fucoidan-induced phosphorylation of ERK1/2 by FPRL1 siRNA transfection in astrocytes

To investigate the effect of FPR, FPRL1 and MARCO on down-regulation of Aβ1–42-, fMLF (FPR- and FPRL1-agonist) and fucoidan-induced signal transduction astrocytes were transfected with FPR, FPRL1 or MARCO siRNA as

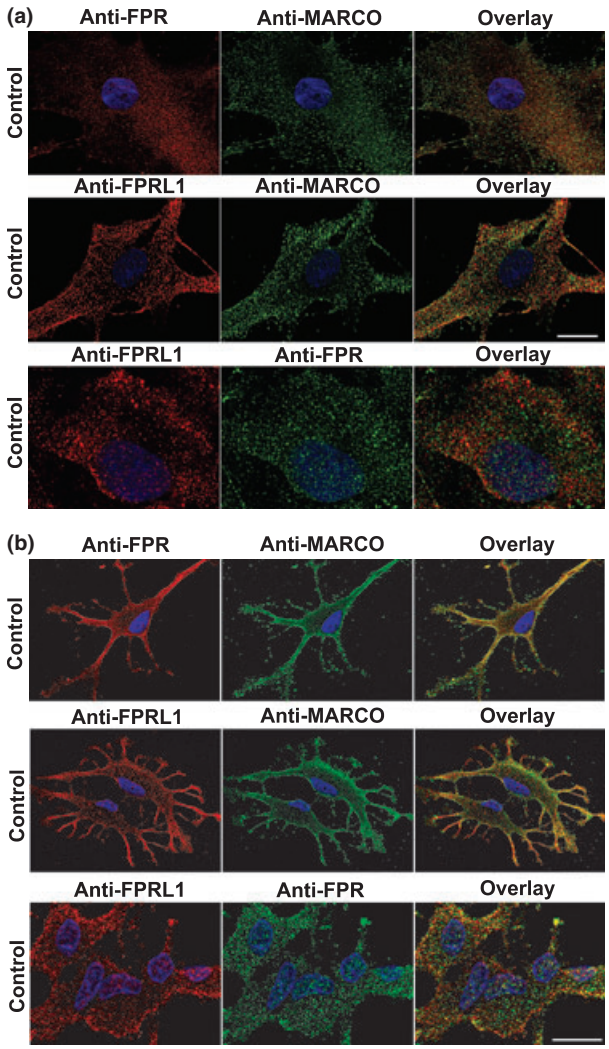

Fig. 3 FPR and FPRL1 are co-localized with MARCO in glial cells. Astrocytes (a) and microglia (b) were fixed and labelled with anti-FPR and anti-FPRL1 or anti-MARCO antibodies. Localization of FPR, FPRL1 and MARCO was examined by double fluorescence microscopy. Bisbenzimide was used for nuclear counter-staining (blue). The figures show representative results from one of three independent experiments, each performed in duplicate. Scale bar: 20 μm.

described above. 96 h after transfection, receptor activity was then determined by ERK1/2 phosphorylation. As shown in Fig. 5(a) and (b), treatment with Aβ1–42, fMLF and fucoidan resulted in an intense phosphorylation of ERK1/2 in astrocytes which had been transiently transfected with control siRNA and also in untransfected control cells (about two- to threefold). Transfection with FPRL1 siRNA completely inhibited ERK1/2 phosphorylation induced by Aβ1–42, fMLF and fucoidan, whereas FPR siRNA showed no effect. Transfection of astrocytes with MARCO siRNA did not change the Aβ1–42- and fMLF-induced ERK1/2 phosphorylation, but inhibited fucoidan did mediate ERK1/2 signalling (Fig. 5a).

MARCO interacts with FPRL1 in transfected HEK293 cells

To confirm our results concerning formyl peptide receptor and MARCO interaction and on the involvement of FPRL1 in fucoidan-induced signal transduction, we stably expressed human FPRL1 (hFPRL1) and MARCO (hMARCO) in HEK293 cells. Co-immunoprecipitation studies were conducted with anti-human FPRL1 antibodies using lysates of hFPRL1-hMARCO expressing HEK293 cells. The precipitates were immunoblotted with antibodies directed against MARCO. As shown in Fig. 6(a), hMARCO was detected in immunoprecipitates from co-transfected HEK293 cells, which confirms that FPRL1 is physically associated with MARCO *in vitro*. Stimulation with Aβ1–42 and fucoidan did not alter the amount of co-immunoprecipitated MARCO in hFPRL1-hMARCO expressing HEK293 cells. As shown in Fig. 6(b), in only hFPRL1, only hMARCO or in mixed hFPRL1 and hMARCO expressing HEK293 cells, we could not detect a co-immunoprecipitation with MARCO.

FPRL1- and MARCO-mediated ERK1/2 phosphorylation and change of cAMP levels in transfected HEK293 cells

In an additional set of experiments, we investigated the effect of FPRL1 and MARCO on Aβ1–42-, fMLF- and fucoidan-induced signal transduction in transfected HEK293 cells. For this purpose, we generated either hFPRL1- or hMARCO-expressing, or hFPRL1 and hMARCO-co-expressing HEK293 cells (for expression levels see Fig. S1(b)), and analyzed ERK1/2 phosphorylation after Aβ1–42-, fMLF- and fucoidan-treatment (Fig. 7a–d). In un-transfected and hMARCO- expressing HEK293 cells, Aβ1–42, fMLF and fucoidan stimulation did not increase the ERK1/2 phosphorylation (Fig. 7a and b). In hFPRL1-transfected HEK293 cells, only Aβ1–42 and fMLF induced ERK1/2 phosphorylation (Fig. 7c), whereas in hFPRL1-hMARCO-expressing HEK293 cells, Aβ1–42, fMLF and fucoidan significantly increased ERK1/2 phosphorylation (Fig. 7d). The results of the western blots were confirmed by densitometric quantification (Fig. 7e–h). The conditions reflected in the data summarized above had an import on the level of Aβ1–42-, fMLF-, and fucoidan-induced changes in forskolin-induced adenylate cyclase activity in transfected HEK293 cells. In fact, as shown in Fig. 7(i) and (l), in HEK293 cells expressing only hMARCO treatment with Aβ1–42, fMLF or fucoidan had no effect on forskolin-induced cAMP accumulation. However, in hFPRL1-transfected HEK293 cells, Aβ1–42 and fMLF attenuated forskolin-induced cAMP accumulation. In hFPRL1-hMARCO-expressing HEK293 cells, treatment with Aβ1–42, fMLF and fucoidan resulted in a reduction of forskolin-induced adenylate cyclase activity (Fig. 7c and d).

Discussion

As substantial interest exists in identifying the cell surface structures that bind and mediate the intracellular effects of

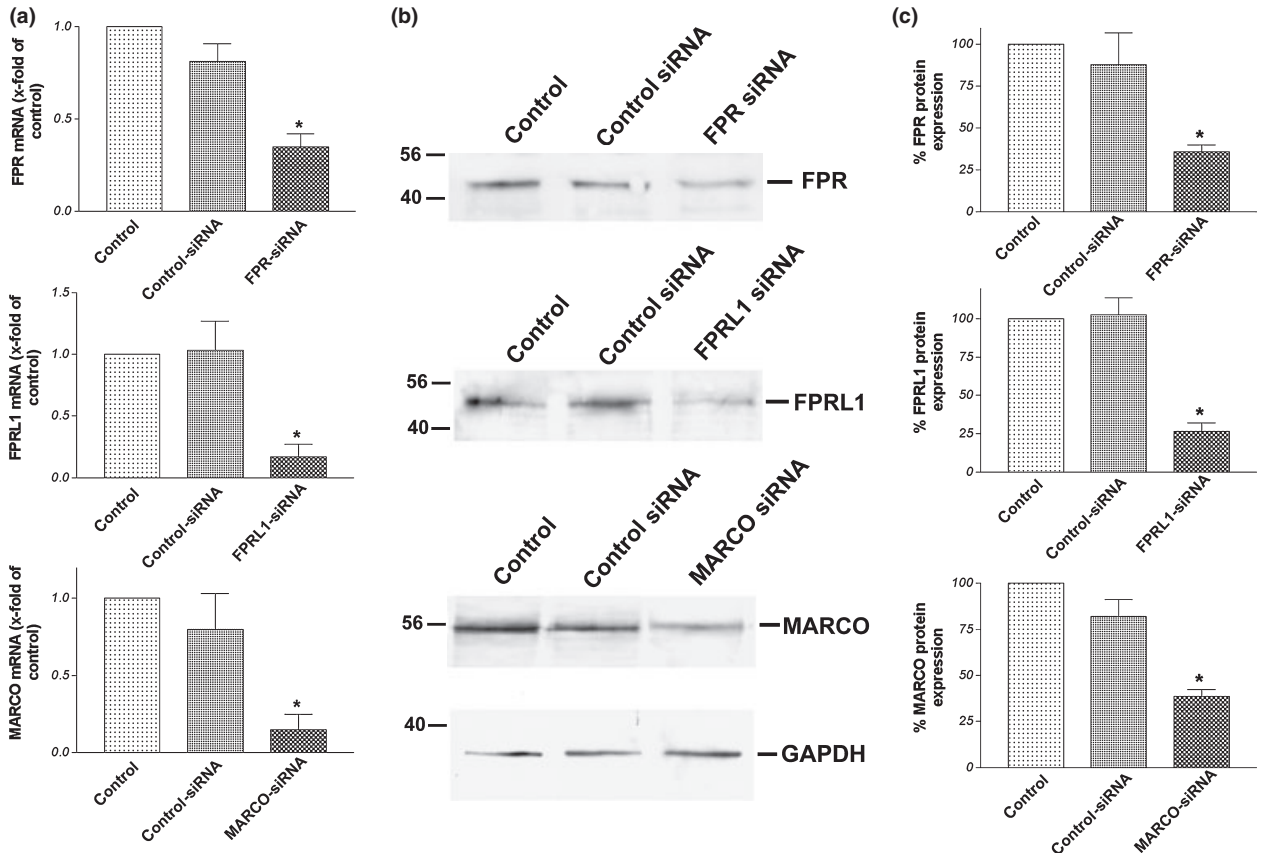

Fig. 4 Inhibition of FPR, FPRL1 and MARCO expression in astrocytes by siRNA. siRNA for FPR, FPRL1 and MARCO as well as control siRNA was transfected in astrocytes and down-regulation of FPR, FPRL1 and MARCO mRNA expression (a) was analyzed 96 h later using SYBR green real time RT-PCR and compared to the untreated sample. GAPDH was used as an internal control (housekeeping gene). Data were assessed from three independent experiments each performed in triplicate. (b) FPR, FPRL1 and MARCO protein expression was evaluated via immunblotting, and two additional experiments produced similar results. The mean ± SD of the three independent experiments was evaluated by densitometric quantification (c). Asterisks indicate a significant difference (*$p < 0.05$) compared to control siRNA (one-way ANOVA followed by the Bonferroni test).

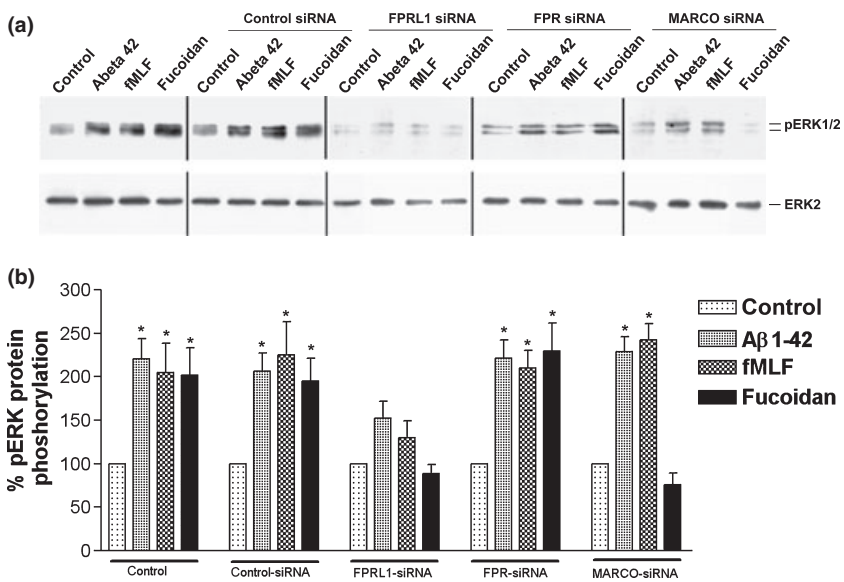

Fig. 5 Inhibition of Aβ1–42 or fucoidan induced ERK1/2 phosphorylation by FPR, FPRL1 or MARCO siRNA transfection in astrocytes. 96 h after transfection, (a) astrocytes were treated with 1 μM Aβ1–42, fMLF or 100 μg/mL fucoidan for 5 min at 37°C. Levels of total ERK2 and phosphorylated ERK1/2 were determined using immunoblotting. The mean ± SD of the three independent experiments was evaluated by densitometric quantification (b). Asterisks indicate significant difference (*$p < 0.05$) compared to control (one-way ANOVA followed by the Bonferroni test).

Aβ1–42, we evaluated the possible participation of two receptor candidates (FPRL1 and MARCO) by monitoring their ability to stimulate Aβ1–42-induced signal transduction. Our group has previously reported on the role of Phospholipase D in endocytosis and signal transduction of Aβ1–42 via the G protein-coupled receptor FPRL1 in glial cells (Brandenburg *et al.* 2008). Other groups have reported that the scavenger receptor MARCO (macrophage receptor with collagenous structure) plays a role in internalization and Aβ1–42-mediated microglia activation (Paresce *et al.* 1996; Koenigsknecht and Landreth 2004). MARCO is a trimeric cell surface glycoprotein with the ability to recognize multiple microbial ligands (Mukhopadhyay *et al.* 2006). MARCO belongs to the class A scavenger receptor molecules. Fucoidan is a ligand for this receptor class (Alarcon *et al.* 2005). Bamberger and colleagues (Bamberger *et al.* 2003) showed that pre-incubation (30 min) of the scavenger receptor MARCO agonist fucoidan inhibited Aβ-stimulated signal transduction and phagocytosis in microglial cells. Further evidence points to an advanced glycation end-product-specific receptor in neurons and microglia cells, RAGE, that binds Aβ1–42 with high affinity (Yan *et al.* 1996; Koenigsknecht and Landreth 2004). As studies have suggested that low-density receptor-related proteins are involved in phagocytosis and in clearance of amyloid beta deposits by glial cells (Arelin *et al.* 2002; Laporte *et al.* 2004), the present study investigated the possibility of formyl peptide receptors (including FPR and FPRL1) and scavenger receptor MARCO in the signal transduction of Aβ1–42 in glial cells.

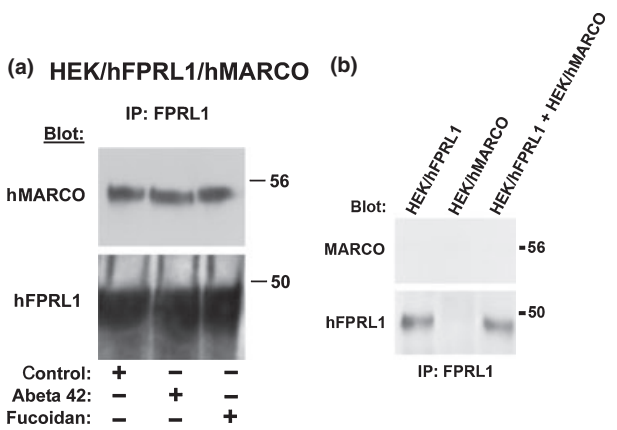

Fig. 6 MARCO interacts with FPRL1 in transfected HEK293 cells. Membrane proteins from **(a)** hFPRL1-hMARCO expressing HEK293 cells with or without 1 μM Aβ1–42 or 100 μg/mL fucoidan pre-treated (30 min) or from **(b)** hFPRL1, hMARCO or mixed hFPRL1 and hMARCO expressing HEK293 cells were extracted and using anti-human FPRL1 antibodies. The resulting immunoprecipitates were electrophoretically separated, transferred to nitrocellulose and detected with anti-FPRL1 or anti-MARCO antibodies. The positions of molecular mass markers are indicated on the right (in kDa). Two additional experiments gave similar results. Human FPRL1 is co-immunoprecipitated with hMARCO in hFPRL1/hMARCO-expressing HEK293 cells. The positions of molecular mass markers are indicated on the right (in kDa). Two additional experiments gave similar results.

As we have previously stated, extracellular-signal regulated kinases 1/2 (ERK1/2) phosphorylation mediates Aβ1–42- and fucoidan-induced signal transduction. This is accompanied by the activation of a G_i-linked receptor, which results in decreased cAMP levels in glial cells (Brandenburg *et al.* 2008; Fig. 1). Studies have shown that fucoidan induces various signal transduction pathways, e.g. protein kinase C, ERK1/2, c-*Jun* N-terminal protein kinase, p38 MAPK or nuclear-factor-κB (Nakamura *et al.* 2006). Deactivation of receptors by antagonists in glial cells or by siRNA experiments in astrocytes verifies the importance of the FPRL1 for Aβ1–42-mediated signal transduction (Figs 1 and 5). Pre-incubation with fucoidan inhibited both the Aβ1–42-induced ERK1/2 phosphorylation and the decrease of forskolin-induced cAMP accumulation, whereas a simultaneous stimulation had no effect on Aβ1–42-induced signal transduction. We conclude that the inhibition of Aβ1–42-induced ERK1/2 phosphorylation subsequent to fucoidan pre-incubation is a consequence of ERK1/2 pathway desensitization, not of a direct receptor antagonism. Notably, the FPRL1 antagonist WRW4 and FPRL1 siRNA inhibited both fucoidan- and Aβ1–42-induced signal transduction (Figs 1 and 5), but whereas siRNA-mediated inhibition of MARCO in astrocytes eliminated fucoidan-induced ERK1/2 phosphorylation, it did not eliminate Aβ1–42-induced ERK1/2 phosphorylation (Fig. 5). FPR siRNA did not show an effect on fucoidan- or Aβ1–42-induced ERK1/2 phosphorylation. A recent study reported that Aβ1–42 is able to activate FPR, but that the receptor's efficacy in mediating cell migration and activation is much lower than that of FPRL1 (Le *et al.* 2001). However, an analysis of FPR and FPRL1 expression (Fig. S1a) clearly shows a lesser expression of FPR in the glial cells, so that we can assume a slight FPR influence on the Aβ1–42 and fucoidan-induced activation.

In this study, we have demonstrated for the first time physical interactions between FPR or FPRL1 and MARCO in astrocytes as well as in microglia by co-immunoprecipitation and fluorescence microscopy (Figs 2 and 3). So far the literature has not described any interaction of formyl peptide receptors with other receptors. Interaction between FPR and MARCO was not affected by Aβ1–42 or fucoidan, whereas interaction between FPRL1 and MARCO increased in glial cells (Fig. 2). The FPRL1-MARCO complex could be important for binding at the cell surface and for subsequent internalization of Aβ1–42. However, our results with transfected HEK cells show that ERK1/2 phosphorylation and inhibition of cAMP level by Aβ1–42 in glial cells is mediated by FPRL1. Further investigations must clarify the importance of the receptor complex for the cell function. In HEK293 cells that over-express hFPRL1 and hMARCO, we were also

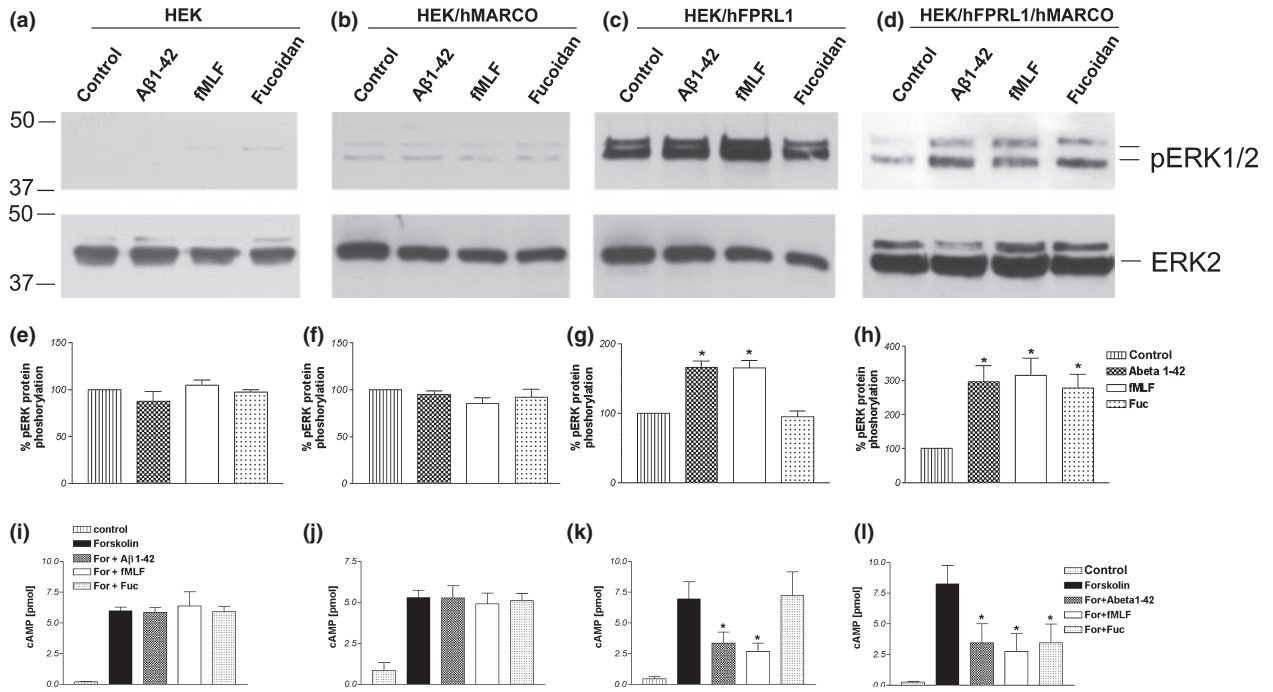

Fig. 7 FPRL1- and MARCO-mediated ERK1/2 phosphorylation and changes of cAMP levels in transfected HEK293 cells. For analysis of ERK1/2 phosphorylation, untransfected (a) or hMARCO- (b), hFPRL1- (c), and hFPRL1/hMARCO- (d) expressing HEK293 cells were treated with 1 μM Aβ1–42, fMLF or 100 μg/mL fucoidan for 5 min at 37°C. Cells were lysed, equal amounts of protein (5 μg) were dissolved by SDS sample buffer, and levels of total ERK2 and phosphorylated ERK1/2 were determined via immunoblotting. The positions of molecular mass markers are indicated on the left (in kDa). The mean ± SD of the three independent experiments was evaluated by densitometric quantification normalized to ERK2 expression (e–h). Asterisks indicate a significant difference (*$p < 0.05$) compared to control (one-way ANOVA followed by the Bonferroni test). For analysis of inhibition of forskolin-stimulated adenylate cyclase activity, untransfected (i) or hMARCO- (j), hFPRL1- (k), and hFPRL1/hMARCO- (l) expressing HEK293 cells were subjected to 25 μM forskolin as well as to 1 μM Aβ1–42, fMLF or 100 μg/mL fucoidan for 15 min at 37°C. cAMP levels were determined as described above (see Experimental Procedure). The values represent mean ± SD from four independent experiments. Asterisks indicate a significant difference (*$p < 0.05$) between forskolin plus agonists or forskolin alone, as determined via one-way ANOVA followed by the Bonferroni test.

able detect an interaction between FPRL1 and MARCO, but this interaction was unaffected by the ligands (Fig. 6). It is possible, because of the high expression of the protein in transfected HEK cells, that no further increase of the interaction quantity after the stimulation with Aβ1–42 or fucoidan is detectable. Bamberger and colleagues (Bamberger *et al.* 2003) have identified a multicomponent receptor for adhesion of Aβ fibrils to microglia as well as subsequent intracellular signal transduction cascades that might not interact with the non-fibrillar form of Aβ. Our results together with those of other groups suggest that FPRL1 mediates the signal transduction and internalization of Aβ1–42 (Le *et al.* 2001; Yazawa *et al.* 2001; Brandenburg *et al.* 2008). Interestingly, both Aβ1–42 and fucoidan induced FPRL1 receptor phosphorylation in both astrocytes and microglia (Fig. 2). Receptor phosphorylation regulates the receptor activation state and also induces β-arrestin binding with subsequent receptor internalization (Potter *et al.* 2002; Vines *et al.* 2003). Besides Aβ1–42, the MARCO ligand fucoidan promotes cross-modulation of FPRL1 phosphorylation and thus influences the activity of this receptor. However, the importance of fucoidan-induced FPRL1 phosphorylation for β-arrestin binding, internalization and glial cell function remains unclear. Further studies must explore this topic.

Based on our results, we hypothesize that FPRL1-bound Aβ1–42 induces signal transduction and is internalized by the receptor (Fig. 8a). The interaction does not affect Aβ1–42 signal transduction by itself. Moreover, we suppose that fucoidan binds to MARCO and that the subsequent signal transduction also depends on FPRL1 (Fig. 8b). In addition, the interaction between FPRL1 and MARCO affects the activity of FPRL1. Our data support the hypothesis that fucoidan-induced interaction with FPRL1 (= transactivation) mediates the activation of MARCO. Consequently, FPR/FPRL1 and MARCO interactions may explain how formyl peptide receptors interact with a menagerie of structurally diverse pro- and anti-inflammatory ligands associated with different diseases including amyloidosis, Alzheimer's disease, prion disease and HIV, or with bacterial components (Le *et al.* 2002). The activation of formyl peptide receptors

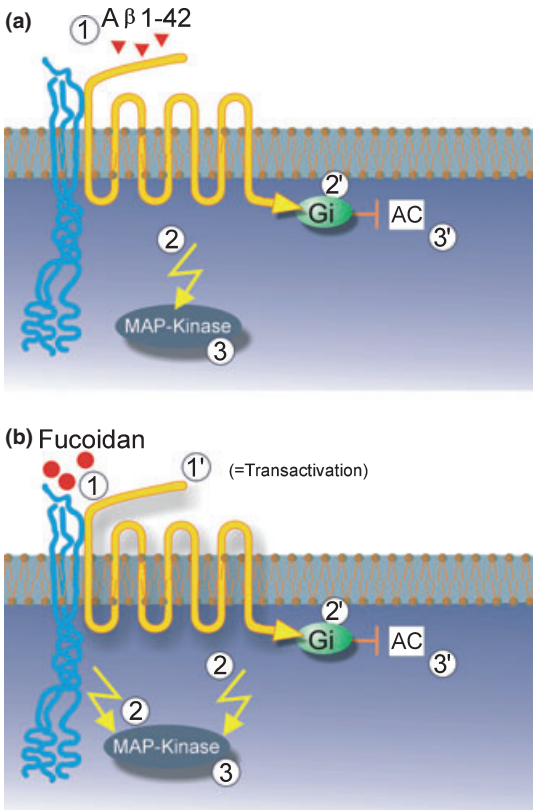

Fig. 8 Model of Aβ1–42- and fucoidan-induced signal transduction in glial cells. (a) Aβ1–42 binds to FPRL1 (yellow, 1), induces signal transduction [2, 3 for MAPKs and 2′, 3′ for adenylate cyclase (AC)] and is internalized by the receptor. The interaction with MARCO (blue) dos not affect Aβ1–42 signal transduction. (b) For fucoidan-induced signal transduction, we hypothesize that fucoidan binds to MARCO, and that the induced signal transduction depends on FPRL1 (2, 3 for MAPKs and 2′, 3′ for AC). Activation of MARCO via fucoidan induced further interaction with FPRL1 and stimulation of signal transduction pathways (1′ = transactivation).

can induce inflammatory signalling including NADPH oxidase and superoxide radical production (Rabiet *et al.* 2007). Our results show that the simultaneous activation of MARCO and FPRL1 leads to a decrease in the Aβ1–42-induced signal transduction in glial cells (desensitization). The interaction between the receptors may also influence the production and secretion of proinflammatory and neurotoxic factors by Aβ1–42. Furthermore, Aβ1–42 internalization into the glial cells with subsequent reduction of pro-inflammatory signalling pathways may thereby be decreased and thus possibly affect the development of the Alzheimer disease. On the other hand the interaction may mediate the Aβ1–42 internalization in glial cells. In any case the interaction might represent an interesting starting point for intervention, and further investigations are necessary. Interactions with other receptors may support and modulate the cellular reaction to such structurally diverse ligands by the formyl peptide receptors. In conclusion, we hypothesize that formyl peptide receptors play a central role in neurodegenerative mechanisms and physiological regulatory processes.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. RT-PCR analysis of receptor mRNA in glial (a) and transfected HEK (b) cells.

Table S1. Oligonucleotide primer sequences used for RT-PCR.

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