Direct binding of a fragment of the Wiskott–Aldrich syndrome protein to the C-terminal end of the anaphylatoxin C5a receptor

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Migration of myeloid cells towards a source of chemoattractant, such as the C5a anaphylatoxin, is triggered by the activation of a G-protein-coupled receptor. In the present study, we have used a yeast two-hybrid approach to find unknown partners of the C5a receptor (C5aR). Using the cytosolic C-terminal region of C5aR as bait to screen a human leucocyte cDNA library, we identified the Wiskott–Aldrich syndrome protein (WASP) as a potential partner of C5aR. WASP is known to have an essential function in regulating actin dynamics at the cell leading edge. The interaction was detected with both the fragment of WASP containing amino acids 1–321 (WASP.321) and WASP with its actin-nucleation-promoting domain [verprolin-like, central and acidic (VCA) domain] deleted. The interaction between C5aR and the WASP.321 was supported further by an *in vitro* binding assay between a radiolabelled WASP.321 fragment and a receptor C-terminus glutathione S-transferase (GST) fusion protein, as well as by GST pull-down, co-immunoprecipitation and immunofluorescence experiments. In the yeast two-hybrid assay, full-length WASP showed no ability to interact with the C-terminal domain of C5aR. This is most probably due to an auto-inhibited conformation imposed by the VCA domain. In HEK-293T cells co-transfected with full-length WASP and C5aR, only a small amount of WASP was co-precipitated with the receptor. However, in the presence of the active form of the GTPase Cdc42 (Cdc42V12), which is thought to switch WASP to an active 'open conformation', the amount of WASP associated with the receptor was markedly increased. We hypothesize that a transient interaction between C5aR and WASP occurs following the stimulation of C5aR and Cdc42 activation. This might be one mechanism by which WASP is targeted to the plasma membrane and by which actin assembly is spatially controlled in cells moving in a gradient of C5a.

Key words: CD88, chemoattractant, Wiskott–Aldrich syndrome protein (WASP), yeast two-hybrid assay.

INTRODUCTION

The migration of leucocytes to sites of inflammation is an essential step in the innate immune response to infection. The interplay of adhesion receptors and chemoattractants that orchestrate this complex process is just beginning to be elucidated (reviewed in [1,2]). Chemoattractants, including N-formyl peptides, C5a anaphylatoxin, and chemokines stimulate a subclass of heptahelical receptors that are linked to heterotrimeric G-proteins of the G_i class (reviewed in [3,4]). In response to G_i-protein activation, leucocytes up-regulate surface adhesion molecules, increase their F-actin content and, even in an uniform concentration of chemoattractant, adopt a polarized morphology, with a trailing uropod and an F-actin-enriched leading edge (reviewed in [2]).

In a gradient of chemoattractant, leucocytes undergo actin-based locomotion towards the source of chemoattractant. The mechanisms by which the direction of the gradient is perceived are not yet clear. Chemotaxis is independent of $G_i\alpha$ but is governed by the release of G_i $\beta\gamma$ subunits [5,6], which activate downstream effectors. These include the γ isozyme of phosphoinositide 3-kinase (PI3Kγ) [7,8] and a guanine-nucleotide-exchange factor for the small GTPases Rac and Cdc42 [9]. PI3Kγ-deficient neutrophils have a reduced motility [10–12] and show a loss of directionality and a random distribution of F-actin in a gradient of *N*-formylmethionyl-leucylphenylalanine (fMLP) peptide [13]. This may result from an inappropriate spatio-temporal activation of the small GTPases Rac and Cdc42, which are both key regulators involved in the reorganization of the actin cytoskeleton and in cell polarity [14,15]. Cdc42 appears to control actin polymerization through the interaction with the GTPase-binding domain (GBD) of the Wiskott–Aldrich syndrome protein (WASP) [16–18], a multidomain protein that is an activator of the actin-nucleating Arp2/3 complex [19]. However, the mechanisms by which WASP is targeted to the leading edge remain unclear.

Chemoattractant receptors are evenly distributed at the surface of cells moving in a chemotactic gradient [20] and yet cells are able to interpret shallow gradients and to navigate to the source of chemoattractant. Moreover, although all chemotactic signals converge on a common chemotaxis-signalling pathway, i.e. the release of G $\beta\gamma$ subunits of G_{i2}, neutrophils are able to interpret competing chemotactic directional signals by responding to the vector sum of the orienting gradients [21]. It is presently unclear if this ability to navigate through complex chemotactic fields involves the agonist-mediated desensitization of chemoattractant receptors, a process implicating the phosphorylation of receptors by G-protein-coupled-receptor kinases (GRKs) and the interaction with β-arrestin scaffolding proteins (reviewed in [22]). In this respect, *in vitro* and *in vivo* studies have given conflicting results. In the case of the chemokine receptors CCR2B and

Abbreviations used: aa, amino acid(s); BR, basic region; C5aR, C5a receptor; DSP, dithiobis(succinimidyl propionate); fMLP, *N*-formylmethionyl-leucylphenylalanine; FPRL1, fMLP-related receptor 1; Gal4AD, activation domain of Gal4; GBD, GTPase-binding domain; GRK, G-protein-coupled-receptor kinase; GST, glutathione S-transferase; HA, haemagglutinin; NP40, Nonidet P40; N-WASP, neuronal WASP; PI3K, phosphoinositide 3-kinase; WASP, Wiskott–Aldrich syndrome protein; WH1, WASP homology 1.

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CCR5, phosphorylation is apparently not a critical step for the chemotactic response [23,24]. However, a recent study of knockout mice indicates that lymphocytes deficient in either β-arrestin 2 or GRK6 are defective in chemotaxis towards a gradient of stromal-cell-derived factor 1 [25].

The transient translocation of modular proteins to chemoattractant receptors in proportion to the level of receptor occupancy might be one of the strategies used by motile cells to sense the direction of a chemotactic gradient. In the present study, we examined, in the case of the C5a receptor (C5aR), whether or not there are possible interactions with partners other than the G_i protein, GRKs and β-arrestins, for a spatial and stoichiometric coupling of the receptor with the actin-polymerization machinery. Using the C-terminal domain of C5aR as bait in the yeast two-hybrid assay, we identified the multidomain protein WASP as a potential partner of C5aR.

EXPERIMENTAL

Two-hybrid screening of a human leucocyte cDNAs library

The cDNA encoding the C-terminal end of the anaphylatoxin C5aR (Val^{301}–Val^{350}) was fused to the cDNA of the LexA DNA-binding protein into the pLEX10 vector, and introduced into the yeast *Saccharomyces cerevisiae* L40 reporter strain. Yeast cells that expressed the LexA–C5aR:301–350 fusion protein were transformed further with cDNAs from a human leucocyte cDNA library in pACT2 vector (Clontech MATCHMAKER), fused to the sequence of the activation domain of Gal4 (Gal4AD). The screen of transformants was based on monitoring the transactivation of two reporter genes (*His3* and *LacZ*). Colonies from 10^5 transformants that were able to grow on selective medium lacking histidine (His^+ phenotype) were then assayed for β-galactosidase activity ($LacZ^+$ phenotype) by a qualitative colour filter assay [26]. The pACT2 plasmids in yeast corresponding to $His^+/LacZ^+$ clones were rescued and amplified in the *Escherichia coli* HB101 strain for further analysis.

Two-hybrid vector constructions and individual assays

The cDNAs encoding residues 301–331, 320–350 and 301–350 of C5aR were amplified by PCR and introduced in the *Eco*RI/*Bam*HI sites of pLex10, in frame with the LexA protein. To obtain serine-mutated constructs in pLex10, we used previously mutagenized C5aR cDNAs [27,28] as PCR templates.

Full-length WASP cDNA was isolated by colony hybridization on to nitrocellulose filters from a HL60 cell library in the CDM8 vector [29]. A 2 kb cDNA was isolated (CDM8-WASP), which started at position -15 of the published 1.8 kb human WASP nucleotide sequence [30]. A *Xho*I/*Xho*I fragment of CDM8-WASP corresponding to the C-terminal 321–502 sequence of WASP was inserted into the *Xho*I site of the pACT2 plasmid that contains WASP amino acids (aa) 1–321 (WASP.321), thus restoring a full WASP coding sequence in pACT2, in frame with that of the Gal4AD protein. PCR was used to retrieve cDNA fragments encoding various truncated WASPs: WASPΔVCA (aa 1–420), WASP.GBD (aa 1–280), WASP.BR (where BR is basic region; aa 1–237), WASP.WH1 (where WH1 is WASP homology 1; aa 1–152), WH1 (aa 39–152) and GBD (aa 223–280). Fragments were ligated into the *Eco*RI/*Xho*I sites of pACT2. The cDNA for WASP.321 depleted from residues 47 to 137 (WASP.321ΔWH1) was obtained by rescuing a 160 bp *Eco*RI/*Msc*I fragment DNA from the pACT2-WASP.321 plasmid and inserting it into the same vector in place of a 430 bp *Eco*RI/*Stu*I fragment DNA.

The ability of Gal4AD–WASP and LexA–C5aR fusion proteins to interact with each other was tested after sequentially transforming the yeast L40 strain with the constructs of interest. In addition to the qualitative His^+ and $LacZ^+$ phenotypic assays, a quantitative method to monitor the expression of β-galactosidase was performed according to [31]. The attenuance of the final supernatant was measured at 420 nm and was normalized to the D_{600} of the initial culture, giving arbitrary units of β-galactosidase expression. Expression of C5aR and WASP fragment proteins in yeast transformants was controlled by immunoblotting yeast lysates with anti-LexA and anti-Gal4AD polyclonal antibodies (Clontech).

Binding of [^{35}S]WASP.321 to a glutathione S-transferase (GST)–C5aR fusion protein

The cDNA encoding WASP.321 was subcloned into the *Eco*RI/*Xho*I sites of pCDNA3.1(+) (Invitrogen) under the control of the T7 RNA polymerase promoter. The [^{35}S]WASP.321 fragment was prepared using the TNT® T7 Coupled Reticulocyte Lysate System kit (Promega), according to the manufacturer's recommendations. The sample was centrifuged through a Micro Bio-Spin 6 chromatography column (Bio-Rad) that was washed with Hepes buffer (30 mM Hepes, pH 7.5, 150 mM KCH_3CO_2 and 2 mM $MgCl_2$). The cDNA encoding the C5aR:301–331 sequence was subcloned into pGEX4T-1 (Amersham Biosciences). The recombinant GST–C5aR:301–331 fusion protein was produced and immobilized on glutathione–Sepharose 4B (Amersham Biosciences) according to standard protocols. Binding assays with the radiolabelled WASP.321 were performed as follows: a minimal amount (5 μl) of sedimented GST–receptor-coupled Sepharose was treated with 2 % (v/v) BSA in buffer B (20 mM Hepes, pH 6.5, 150 mM NaCl, 2 mM EDTA, 3 mM $MgCl_2$ and 1 mM dithiothreitol). BSA was discarded and the beads were incubated further with 5 μl of [^{35}S]WASP.321 (50 000 cpm, estimated at 350 fmol) at room temperature (20 °C) for 2 h. Beads were washed three times with 1 ml of buffer B. Analysis of the amount of [^{35}S]WASP.321 bound was performed by SDS/PAGE (10 % gel) and PhosphorImager.

Cell culture and cell lines

Cell lines were cultured at 37 °C in humidified atmosphere of air/CO_2 (19:1), in Dulbecco's modified Eagle's medium/F12 with GlutaMAX I (Invitrogen) for COS-7 and HEK-293T cells or RPMI 1640 medium with GlutaMAX I supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % (v/v) heat inactivated foetal calf serum (Invitrogen) for RINm5F cells. COS-7 cells and HEK-293T cells were transiently transfected by a standard calcium phosphate procedure. The stable expression of wild-type C5aR in the rat insulinoma cell line RINm5F has been described previously [27].

Glutathione–Sepharose pull-down assay

The cDNA of WASP.321 was subcloned into pGEX4T-2. The resulting GST–WASP.321 fusion protein was produced and immobilized on glutathione–Sepharose 4B, according to standard protocols. In C5aR pull-down assays, C5aR-expressing RIN cells (2×10^6 cells) were either not stimulated or stimulated with 100 nM human recombinant C5a (Sigma) for 10 min at 37 °C. Stimulation was stopped by lysis in 1 ml of ice-cold buffer B in the presence of 1.2 % (v/v) Nonidet P40 (NP40), protease inhibitors [10 μg/ml leupeptin, 10 mM benzamidine, 5 μM

pepstatin, 0.2 μg/ml aprotinin, 10 μg/ml *p*-toluenesulphonyl-L-arginine methylester hydrochloride, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride] and phosphatase inhibitors (50 nM calyculin A, 10 mM NaF and 1 mM *p*-nitrophenyl phosphate). The resulting homogenate was centrifuged at 800 ***g*** for 5 min at 4 °C to remove unbroken cells and nuclei. WASP.321-immobilized beads were previously incubated in the presence of 2 % (v/v) BSA in lysis buffer at room temperature for 30 min. The BSA-containing supernatant was discarded, cell lysates were added and the incubation was continued overnight at 4 °C. The beads were washed rapidly three times with 1 ml of cold lysis buffer and resuspended in 2× Laemmli buffer for analysis of the amount of C5aR bound.

Immunoprecipitation and Western blot analysis

Transfected HEK-293T cells in 10 cm diameter culture dishes were first treated with 2.5 mM dithiobis(succinimidyl propionate) (DSP; Pierce) for 35 min at 30 °C. After gentle washes in PBS and then in ice-cold buffer T (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1 mM EGTA), cells were lysed in buffer T in the presence of 1 % (v/v) NP40 and protease and phosphatase inhibitors (as above). Immunoprecipitation of C5aR was performed by incubating the clarified lysates with an affinity-purified anti-peptide antibody directed against residues 339–350 of C5aR [32], then by further incubation with Protein A–Sepharose. To reduce non-specific binding, Protein A–Sepharose was pre-incubated overnight in PBS supplemented with 2 % (v/v) BSA. The beads were washed three times with ice-cold lysis buffer and once with PBS. The amount of C5aR or WASP present in the immunoprecipitates was analysed by Western blotting. Samples were denatured by heating at 50 °C for 30 min in 2× Laemmli buffer in the presence of 25 mM dithiothreitol, then subjected to SDS/PAGE (10 % gel) and electrotransferred on to nitrocellulose. Immunodetection was performed in a blocking buffer [PBS containing 0.1 % (v/v) NP40 and 3 % (w/v) low-fat dry milk powder] using the anti-C5aR:301–350 antibody or an affinity-purified polyclonal anti-WASP antibody raised against the peptide SSRYRGLPAPGPSPADKKRS[228] (BioCytex, Marseille, France). Final detection was performed as indicated in the Figure legends.

Immunofluorescence

COS-7 cells (10^5 cells) were seeded on polylysine-treated coverslips 24 h before transfection. Cells were transiently transfected with 0.5 μg of either pCDNA3-WASP.321 or CDM8-WASP plasmids along with a haemagglutinin (HA)-tagged C5aR-encoding pCDNA3 plasmid or mock pCDNA3 (0.5 μg). At 48 h after transfection, cells that expressed WASP and/or HA-tagged receptor were cultured for 3 h in the presence of 100 μg/ml cycloheximide, then stimulated in the presence or absence of 100 nM C5a for 10 min at 37 °C. Cells were washed with serum-free RPMI 1640 medium and permeabilized by immersion in acetone at −20 °C for 30 s. Immunostaining of C5aR was performed with an anti-HA monoclonal antibody (clone 12CA5; Roche) and that of WASP with the polyclonal anti-WASP antibody. Secondary antibodies were BODIPY® FL-conjugated goat anti-mouse IgG and Alexa Fluor® 568-conjugated goat anti-rabbit IgG (Molecular Probes). Images for the median plane of cells were acquired with an oil immersion ×63 objective of a Leica TCS-P2 confocal scanning microscope. Laser excitation wavelengths were set at 488 nm and 543 nm.

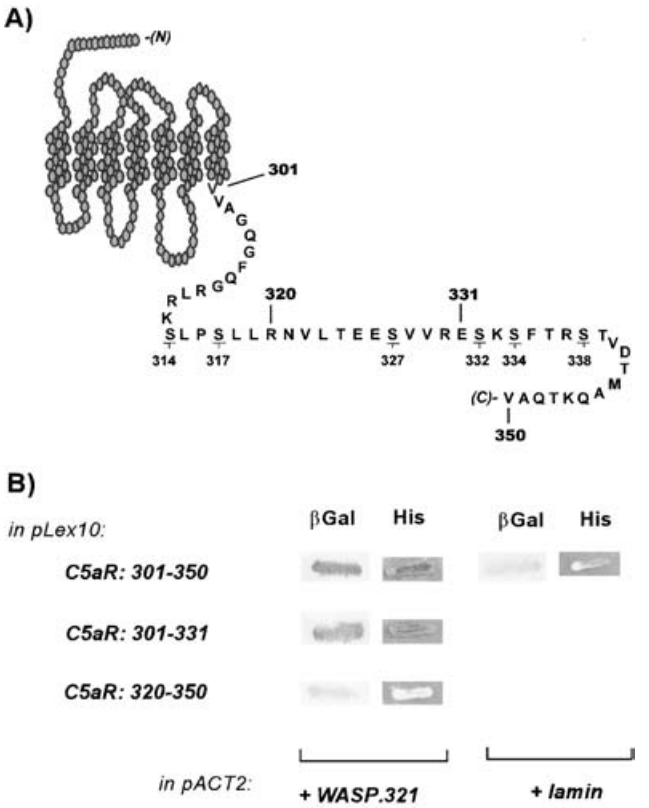

Figure 1 Interaction of the C5aR C-terminal tail with a fragment of WASP

(**A**) The seven-membrane-spanning anaphylatoxin C5aR. The sequence of the cytosolic C-terminal end is indicated in full, using the one-letter aa code. Serine residues that become phosphorylated upon C5a stimulation are underlined and numbered. Numbers in bold indicate termini positions of the constructs in the yeast two-hybrid pLex10 vector, corresponding to full-length (301–350), and membrane proximal (301–331) and distal (320–350) portions of the C-terminal tail. (**B**) Two-hybrid assays between the different constructs of the C5aR C-terminus and WASP.321. Lamin, instead of WASP.321, was inserted in the two-hybrid vector pACT2 to control the specificity of the interaction. βGal, colour-filter assay for β-galactosidase activity; His, growth on histidine-deficient medium.

RESULTS

A fragment of WASP interacts with the cytosolic C-terminal tail of the C5aR in yeast and *in vitro*

To identify potential C5aR-interacting proteins, we used the two-hybrid approach with the C-terminal end of C5aR (Val^{301}–Val^{350}; Figure 1A), fused to the LexA protein, as bait to screen a human leucocyte cDNA library. Of the histidine- and β-galactosidase-positive clones, half contained an approx. 1 kb cDNA insert that encoded the N-terminal two thirds (residues 1–321) of the WASP, a 502-aa-long protein that is involved in the regulation of actin polymerization in myeloid cells. Regions of the C5aR C-terminus were tested individually against WASP.321. The segment that is proximal to the plasma membrane was tested positive (C5aR:301–331), whereas the distal portion (C5aR:320–350) completely failed to interact with WASP.321 (Figure 1B). Immunoanalysis revealed that the expression levels of the LexA–C5aR:301–331 and LexA–C5aR:320–350 fusion proteins in yeast were at least 10-fold higher than that of the LexA–C5aR:301–350 fusion (results not shown), ensuring that the failure of the terminal 320–350 portion to interact with WASP.321 was not due to defective expression. The overlapping fragment 320–331 may have little, if any, contribution to the interaction.

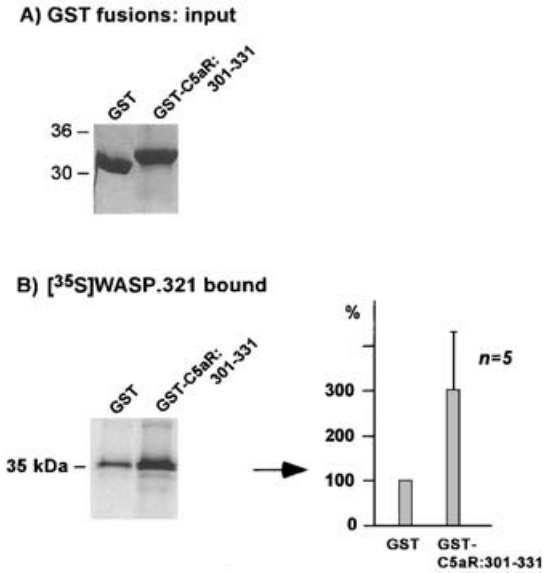

Figure 2 Direct *in vitro* binding of [^{35}S]WASP.321 to the C5aR C-terminal end

The membrane proximal portion of the C5aR C-terminal end (C5aR:301–331) was expressed as a GST fusion protein and mixed to *in vitro* synthesized [^{35}S]WASP.321 (50 000 cpm, approx. 350 fmol). (**A**) GST input: Coomassie Blue staining of the amount of Sepharose-coupled GST (control) and Sepharose-coupled GST–C5aR:301–331 fusion protein that is used per assay. (**B**) SDS/PAGE (10 % gel) analysis of the amount of [^{35}S]WASP.321 bound to the Sepharose-coupled GST fusion protein. Radioactivity bound was visualized and quantified using the PhosphorImager. The radioactivity bound to GST alone (background) was set to 100 %.

To assess further the association between WASP.321 and C5aR, *in vitro* binding experiments were performed between the ^{35}S-radiolabelled WASP.321 fragment and the GST–C5aR:301–331 fusion protein (Figure 2). Based on SDS/PAGE (10 % gel) with Coomassie Blue staining, an equal amount of Sepharose-coupled GST protein was used as control in a parallel binding experiment (Figure 2A, input). Despite important variations in the extent of binding between independent experiments, the amount of [^{35}S]WASP.321 precipitated along with the GST–C5aR C-terminus was consistently 2- to 3-fold higher than the amount bound to GST alone (a mean of 301 ± 126 % of control, $n = 5$; Figure 2B). The association between WASP.321 and C5aR could thus occur without the need for any cellular intermediate.

The WASP.321 fragment interacts with membrane-embedded C5aR

In the context of membrane-expressed receptor, the protein sequence used as bait in the two-hybrid approach is submitted to structural constraints that are quite different from those in the LexA fusion protein. Furthermore, ligand binding induces conformational changes of the cytosolic C-terminal end of C5aR, followed by phosphorylation on serine residues [33], which might deeply affect the interaction of potential partners with the receptor.

To examine whether the WASP.321–C5aR interaction occurred with full-length C5aR and to what extent this interaction was dependent on receptor activation, we tested further the ability of the GST–WASP.321 fusion protein to pull-down C5aR from lysates of cells either treated or untreated with C5a. Lysates were incubated with glutathione–Sepharose beads coupled to either GST alone or to the GST–WASP.321 fusion protein. C5aR in lysates (Figure 3, left panel) or precipitated along with the beads (Figure 3, right panel) was analysed by immunoblotting with a polyclonal anti-peptide antibody against the C-terminus of

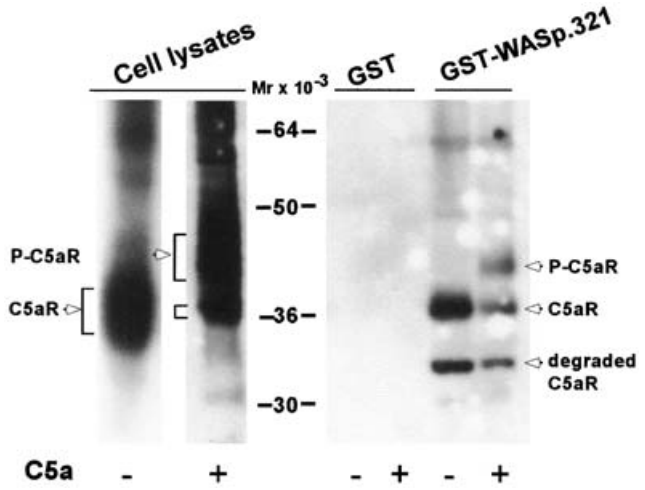

Figure 3 C5aR pull-down assay

RINm5F cells that stably expressed the C5aR were either not stimulated or stimulated with 100 nM C5a for 10 min at 37 °C. Lysates from these cells were incubated with GST alone (control) or with the GST–WASP.321 fusion coupled to glutathione–Sepharose beads. An equal amount of matrix and of bound GST proteins was standardized between the GST–WASP.321 and the GST samples. An aliquot (1/30) of lysates (left panel) and the total Sepharose-bound receptor (right panel) were analysed by SDS/PAGE (10 % gel) and Western blotting using a polyclonal anti-C5aR antibody directed against the C-terminal peptide. Peroxidase-conjugated Protein A and enhanced chemiluminescence (ECL®) detection system from Amersham Biosciences were used for final detection. P-C5aR, phosphorylated C5aR.

C5aR. As shown previously [27], in response to C5a binding, a large fraction of C5aR was shifted from the non-phosphorylated form that migrated with a molecular mass of approx. 40 kDa to the phosphorylated species that migrated with an apparent molecular mass of approx. 45 kDa (Figure 3, left panel). Analysis following the pull-down assay revealed that both non-phosphorylated and phosphorylated forms of C5aR were able to interact specifically with WASP.321 (Figure 3, right panel). However, when comparing the amount of receptor precipitated by GST–WASP.321 with the initial amount of C5aR present in lysates, more non-phosphorylated C5aR was consistently recovered. The interaction between WASP.321 and C5aR thus appeared to occur preferentially with unstimulated receptors.

Co-immunoprecipitation of WASP.321 and full-length WASP with C5aR

To determine whether C5aR formed a complex with WASP.321 and full-length WASP in cells, HEK-293T cells were transiently co-transfected with the receptor and either WASP.321 or full-length WASP. Before lysis, cells were treated with DSP to cross-link C5aR-associated proteins and the receptor was immunoprecipitated using an antibody directed against the C-terminus (Figure 4). After cross-linking, the receptor was immunoprecipitated efficiently (Figure 4A, left panel, lane 4) and, when WASP.321 was co-expressed with the receptor, a small fraction of WASP.321 was pulled-down with C5aR (Figure 4A, right panel, lane 4). Co-immunoprecipitation of WASP.321 was not due to non-specific binding to immunoglobulins or to Sepharose beads since WASP.321 was not detected in immunoprecipitates from cells that were not transfected with C5aR (Figure 4A, right panel, lane 2). When full-length WASP was co-transfected with C5aR, only a small amount of WASP was immunodetected in a complex with C5aR (Figure 4B, lane 7). WASP was undetectable when cells were not

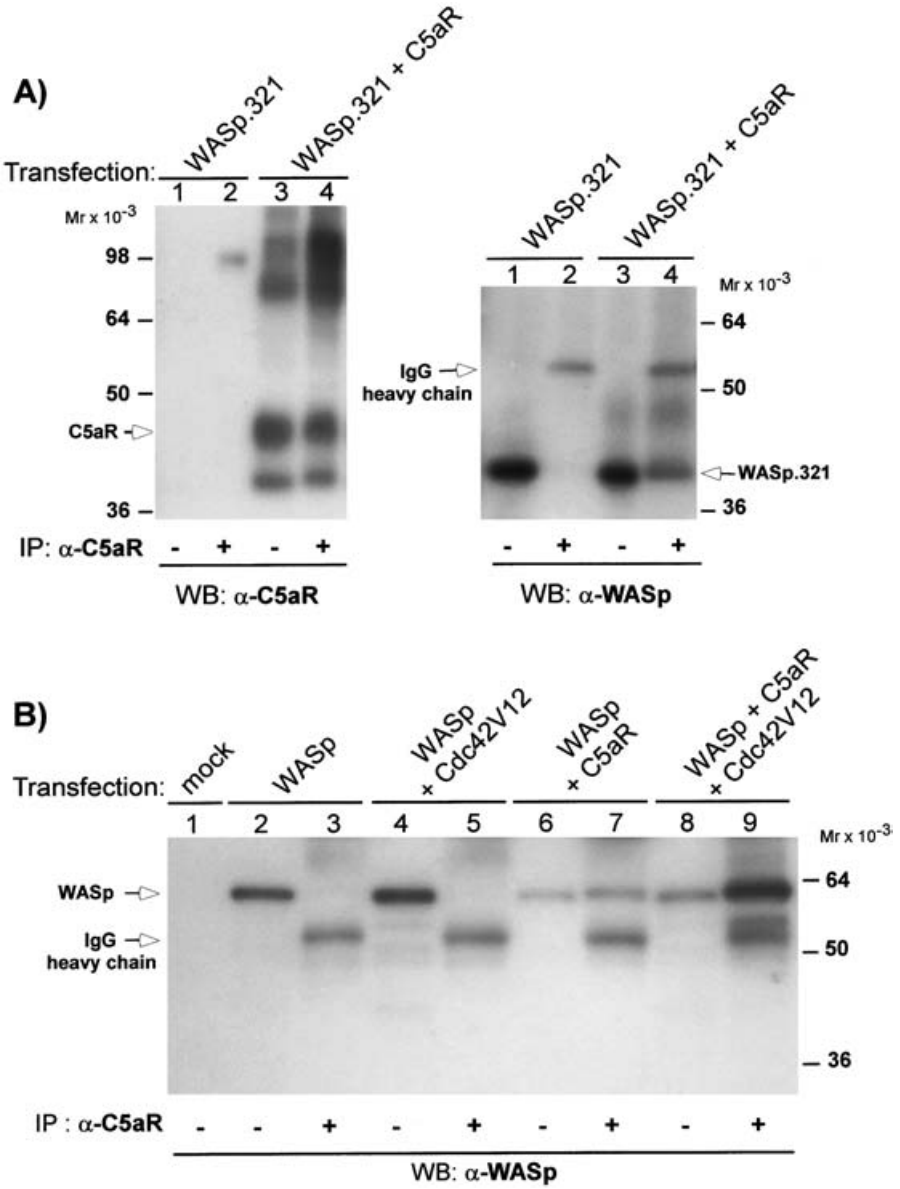

Figure 4 Detection of the association of WASP.321 and full-length WASP with C5aR in HEK-293T cells

Cells were transiently transfected and, before lysis, receptor complexes were stabilized by cross-linking with DSP. Immunoprecipitations of complexes were performed with an antibody directed against the last ten residues of C5aR. (**A**) Left panel: immunodetection of C5aR representing 50 % of the amount immunoprecipitated from the whole-cell lysates (lanes 2 and 4) or representing an aliquot (2 %) of the lysates (lanes 1 and 3). Right panel: immunodetection representing 50 % of the amount of WASP.321 co-precipitated with C5aR (lanes 2 and 4) or representing the amount of WASP.321 present in an aliquot (2 %) of the lysates. (**B**) Immunodetection of full-length WASP present in an aliquot (1 %) of whole-cell lysates (lanes 1, 2, 4, 6 and 8) or co-precipitated with C5aR (lanes 3, 5, 7 and 9). Radio-iodinated recombinant protein A/G was used for final detection. The results presented are representative of three independent experiments. IP, immunoprecipitation; WB, Western blot.

transfected with the receptor, indicating that WASP was associated specifically with the receptor (Figure 4B, lane 3). The amount of WASP co-precipitated with C5aR was markedly increased when cells were co-transfected with the activated mutant form of Cdc42 (Cdc42V12) as illustrated in Figure 4(B) (lane 9). This increase is unlikely to result from a Cdc42V12-mediated non-specific interaction with the immunoglobulins or the Sepharose beads since WASP was not detected in the absence of C5aR expression (Figure 4B, lane 5). No significant difference could be detected when cells were briefly stimulated with C5a before the addition of DSP (results not shown). Thus full-length WASP appears to interact more efficiently with C5aR when its constrained, auto-inhibited structure is switched to an 'open conformation' through the interaction with the activated form of Cdc42 [34–36].

C5aR expression modifies the cellular distribution of WASP.321

Only a minor fraction of endogenous WASP is present at the plasma membrane of haematopoietic cells, while the major pool resides in the cytosol [37,38]. Overexpression of either WASP or its C-terminal functional domain in transfected cells leads to the formation of actin clusters in the cytosol [18,39]. However, several authors have proposed that WASP recruitment

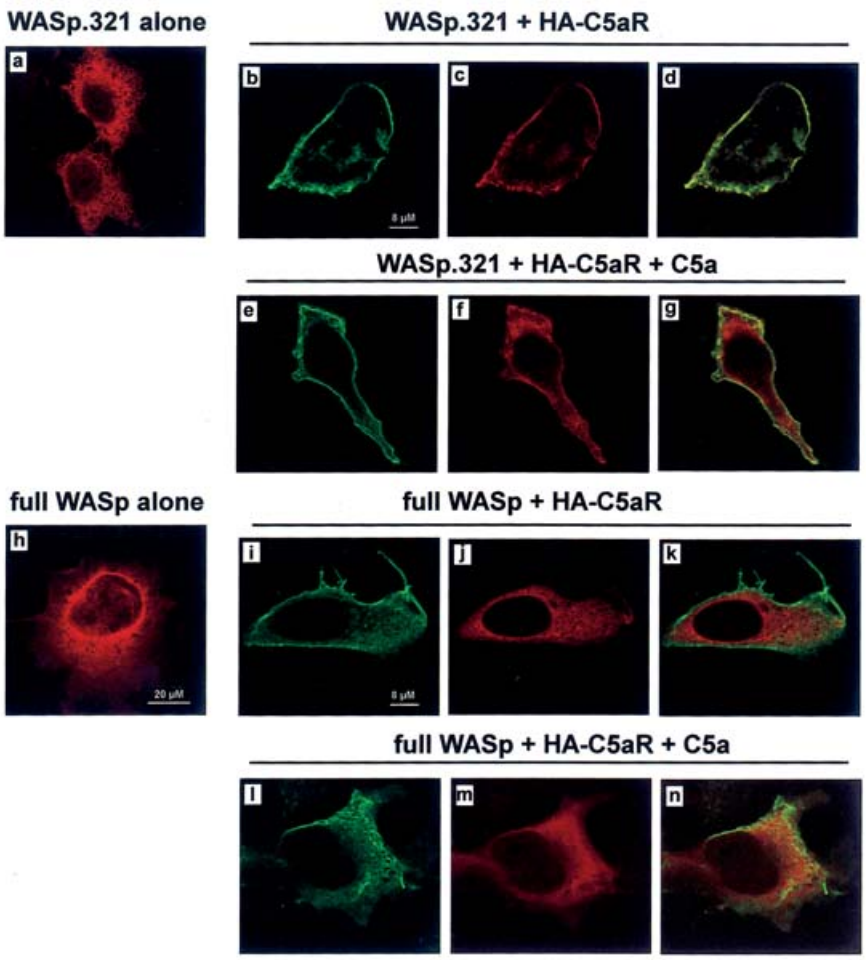

Figure 5 Effect of C5aR expression on WASP.321 and WASP localization in COS-7 cells

Recombinant WASP.321 and full-length WASP were detected by immunofluorescence staining of transfected cells either treated or not treated with C5aR. COS-7 cells were transiently transfected with WASP.321 (panels a–g) or full-length WASP (panels h–n) cDNA, either alone (panels a and h) or in combination with HA-tagged C5aR cDNA. In panels e–g and l–n, cells were challenged with C5a before fixation in acetone. Panels a, c, f, h, j and m (red staining) represent Alexa Fluor® 568-staining of WASPs interacting with the polyclonal anti-WASP antibody. Panels b, e, i and l (green staining) represent BODIPY ® FL-staining of C5aR interacting with the monoclonal anti-HA antibody. Panels d, g, k and n are merged images.

at the cell membrane is a physiological step required to elicit its actin-nucleating activity [35,40]. Factors that contribute to the docking of WASP at the plasma membrane are not well understood. We used an immunofluorescence staining approach to confirm further that the expression of C5aR contributes to the localization of WASP at the plasma membrane. COS-7 cells were transfected with either WASP.321 or full-length WASP, alone or in association with HA-tagged C5aR. In the absence of C5aR expression, recombinant WASP.321 and full-length WASP were immunodetected in the cytosol mainly around the nucleus (Figure 5, panels a and h respectively) in accordance with previous studies [18,39,41]. This was particularly striking for full-length WASP that formed aggregates, whereas WASP.321 had a more diffuse cytosolic distribution. In each case, there was no staining of the cell membrane. In contrast, in cells that co-expressed C5aR and WASP.321 (Figure 5, panels b, c and d), the antibody directed against WASP markedly stained the plasma membrane (Figure 5, panel c) and the superimposition of the confocal images suggested that WASP.321 co-localized with C5aR at the cell surface (Figure 5, panel d). When these cells where challenged with C5a, a peripheral distribution of WASP.321 was still observed, but co-localization with C5aR at the plasma membrane was less striking (Figure 5, panels e, f and g). This observation is consistent with the result obtained in the GST pull-down assay, suggesting that WASP.321 binds less efficiently to phosphorylated C5aR. It should be noted that C5aR is internalized inefficiently in COS7 cells, in spite of a correct phosphorylation ([28] and F. Boulay, M. Tardif and T. Christophe, unpublished work). When full-length WASP was co-expressed with C5aR (Figure 5, panels i–n), it was distributed more homogeneously throughout the cytosol, but did not redistribute to the plasma membrane (Figure 5, panels j and m) and co-localization with the receptor was not detectable anywhere in the cell (Figure 5, panels k and n), whether or not cells were stimulated with C5a. The lack of WASP translocation to the plasma membrane may result from the inability of agonist-occupied C5aR to trigger signalling events, namely the activation of small GTPases. Indeed, previous studies have shown that chemoattractant receptor-mediated signalling is deficient in COS-7 cells unless cells are co-transfected with the α subunit of G_{16} [42–44].

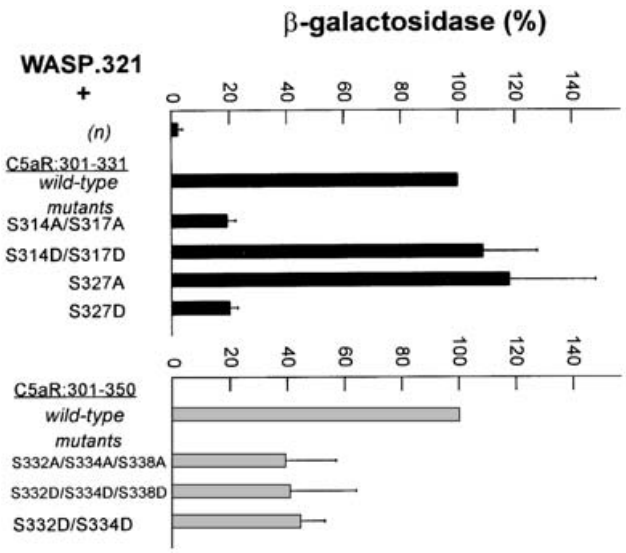

Figure 6 Two-hybrid interactions of serine-modified C5aR C-terminal tail with WASP.321

Two-hybrid interaction assays between the serine-mutated C-terminal ends of C5aR and WASP.321. The various phosphorylation C5aR mutants were constructed in pLex10 vector on the basis of the full-length receptor C-terminal tail (aa 301–350, grey bars) or of the proximal part (aa 301–331, black bars). The β-galactosidase-expression level was independently standardized in each series, as the percentage of the level of expression obtained with the respective wild-type C5aR construct. Results are means $\pm$ S.E.M. from at least 4 independent experiments. *n*, pLex10 vector with no insert. The positions of mutated serine residues are shown in Figure 1.

Effects of mutations in the C-terminus C5aR on the interaction with WASP in yeast

In view of the preferential pull-down of the non-phosphorylated form of C5aR, we analysed the potential impact of serine phosphorylation by using a two-hybrid strategy. Serine residues were replaced with either alanine to generate a bait that mimics the C-terminal end of a phosphorylation-deficient receptor [28] or with negatively charged aspartic acid residues to generate a bait that mimics the phosphorylated receptor. In mammalian cells, C5a mediates the phosphorylation of the receptor carboxylic tail at positions 314, 317, 327, 332, 334 and 338 (see Figure 1A) [28,33]. Phosphorylation of the membrane proximal residues (Ser^{314}, Ser^{317} and Ser^{327}) that belong to the region that interacts with WASP.321 might be directly involved in the interaction, while phosphorylation of the three distal residues (Ser^{332}, Ser^{334} and Ser^{338}) could indirectly regulate the interaction by inducing a conformation change of the C-terminus [28]. The serine replacements had no effect on the level of receptor fusion expression in yeast (results not shown), allowing quantitative interaction assays between WASP.321 and the LexA–C5aR fusion mutants of both the membrane proximal region (C5aR:301–331) and the full C-terminal region (C5aR:301–350) (Figure 6, black bars and grey bars respectively).

The conversion of Ser^{314} and Ser^{317} into alanine (S314A/S317A) diminished the β-galactosidase signal up to 80%, whereas their conversion into aspartate (S314D/S317D) had roughly no effect. On the contrary, mutation of a negatively charged residue at Ser^{327} to aspartate (S327D) dramatically reduced the interaction signal while the presence of alanine (S327A) had no effect or tended to slightly increase the level of β-galactosidase signal above that of the corresponding wild-type construct (wild-type; C5aR:301–331; Figure 6, black bars). The latter pattern suggests that phosphorylation of Ser^{327} could be a signal by which the interaction between C5aR and WASP.321 is regulated. The pattern obtained with Ser^{314} and Ser^{317} is ambiguous to interpret. If one assumes that the two serine residues are not phosphorylated constitutively in a yeast context, it is less their phosphorylation status than the nature of the residue at positions 314 and 317 that is important for the interaction with WASP.

The clear-cut effects observed with the proximal serine residues were not observed with the distal ones (Figure 6, grey bars). Mutants at positions 332, 334 and 338, regardless of the type of replacements A or D (S332A/S334A/S338A, S332D/S334D and S332D/S334D/S338D) displayed a signal that was reduced by approx. 50% compared with that observed with the corresponding wild-type construct (wild-type C5aR:301–350). The relevant observation is that mutation of the distal serine residues into aspartate had no potentializing effect compared with alanine mutants or the wild-type. Thus, despite the fact that the phosphorylation of these serine residues has been shown to release a conformational constraint (i.e. favouring the access of the membrane proximal region to kinases [28]), it does not appear to facilitate the access to WASP.321. As a whole, mimicking various phosphorylation states of the receptor in yeast never favoured the binding of WASP.321.

Mapping of the binding site of C5aR C-terminal tail within WASP

All of the WASP-positive clones identified from the two-hybrid screen were of the same length (aa 1–321). This probably arose from the conjunction of two factors: (i) the presence of a *Xho*I restriction site in the WASP cDNA sequence corresponding to aa 321, and (ii) the fact that the cDNA library from Clontech was inserted in the pACT2 vector using a *Xho*I-based adapter. We therefore tested full-length WASP, as well as several other WASP constructs, to map the domain that potentially binds to or modulates binding to C5aR. WASP is a modular protein schematically represented in Figure 7(A). The partial sequence (WASP.321) that was found to interact with C5aR encompasses about two thirds of the protein. This includes the WH1 domain, the GBD and a small stretch of proline residues. Most of the polyproline-rich ('PPr') region and the Arp2/3 activating domain [or verprolin-like, central and acidic (VCA) domain] were excluded from WASP.321. Using a quantitative β-galactosidase assay, a series of WASP constructs (Figure 7B) was tested against the full-length receptor tail (C5aR:301–350) or against its membrane proximal portion (C5aR:301–331) (Figure 7C). Strikingly, full-length WASP did not interact with any of the receptor C-termini (Figure 7C, lanes a). WASP has been shown to adopt an intramolecular folded structure where a GBD-overlapping sequence ('autoinhibitory region', Figure 7A) interacts with and masks the actin-nucleation-activating domain, i.e. VCA. In this conformation, the VCA domain is unable to activate Arp2/3 [35,36,39]. Such a folding could occur in yeast and impair the interaction between full-length WASP and the receptor bait. Accordingly, when performing the assay with WASP lacking the VCA domain (WASPΔVCA), interactions with the C5aR C-termini were recovered (Figure 7C, lanes b). The intensity of the signal obtained with WASPΔVCA was however consistently below that obtained with WASP.321. The ability of WASPΔVCA to interact with the C5aR C-terminal tail is consistent with the result obtained in the co-immunoprecipitation assay, suggesting that WASP has to be switch to an 'open conformation' to allow the interaction with C5aR.

The WH1 domain of WASP is similar to the EVH1 [Ena/vasodilator-stimulated phosphoprotein (VASP) homology 1] domains, which bind to short proline-rich consensus motifs [45]

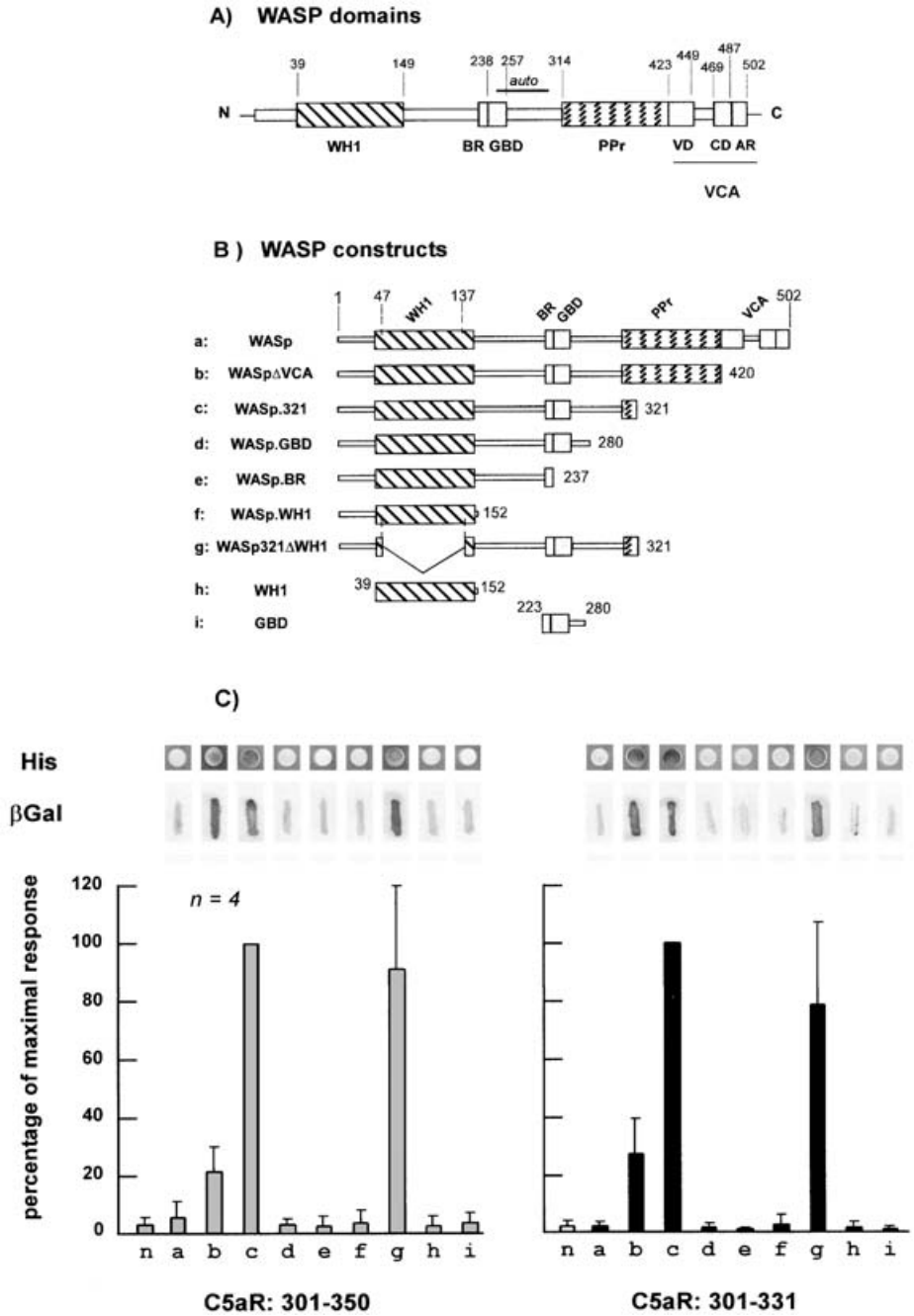

Figure 7 Two-hybrid interactions of C5aR C-terminal tail with various WASP constructs

(**A**) WASP structural domains: WH1, WASP homology 1 domain; BR, basic region; GBD, G-protein-binding domain; PPr, polyproline-rich region; VD, verprolin domain; CD, cofilin domain; AR, acidic region; *auto*, autoinhibitory region. VD, CD and AR are together referred to as the VCA domain. (**B**) WASP constructs were designed in pACT2 vector. (**C**) L40 yeast cells were co-transformed with the pACT2 plasmid encoding one of the various WASP constructs [a–i letter code referring to constructs in (**B**)] and the pLex10 plasmid coding for either the full-length (C5aR:301–350) or the proximal part (C5aR:301–331) of the receptor carboxylic end. n, pACT2 vector with no insert. At least four independent quantitative β-galactosidase assays were performed. β-Galactosidase activity is represented as a percentage of the highest response in each series, obtained here with WASP.321 (lanes c).

and are found in both a number of cell-surface receptors [46] and an extended number of protein families [47]. However, no clear consensus ligand sequence for the WH1 domain of WASP proteins has been determined at the present time. Thus, even though the C5aR C-terminal tail does not contain polyproline motifs, we examined whether the WH1 domain of WASP could be the region of interaction with C5aR. As illustrated in Figure 7(C), neither the WH1 domain (residues 39–152; Figure 7C, lanes h) itself nor the truncated forms of WASP.321 that still included the WH1 domain (WASP.GBD, WASP.BR and WASP.WH1) (Figure 7C, lanes d, e and f respectively) interacted with the C-terminal tail of the receptor. In addition, the WH1-deleted construct (WASP.321ΔWH1) was able to interact with the receptor baits as efficiently as intact WASP.321 (Figure 7C, lanes g). These results show that the WH1 domain is not necessary for the physical association between WASP and C5aR which could mean that,

in vivo, the WH1 domain remains available for other partners, such as WASP-interacting protein ('WIP') [48–50]. Conversely, the short sequence downstream of GBD (residues 280–321) seemed to be essential for the binding to the receptor, since WASP.321 that lacked this sequence (WASP.GBD construct) completely failed to interact with the receptor bait (Figure 7C, lanes d), in spite of an expression level similar to that of WASP.321 (results not shown).

DISCUSSION

The mechanisms by which cells are able to sense a gradient of chemoattractant are still unclear. The asymmetrical distribution of PtdIns(3,4,5)P_3 and F-actin at the leading edge of motile cells [51], together with the loss of directionality and reduced mobility of cells lacking PI3Kγ [13], has led to the hypothesis that the PI3Kγ-mediated formation of PtdIns(3,4,5)P_3 at the leading edge is part of the cellular compass that directs chemotaxis [52]. However, this hypothesis does not completely explain how cells detect a gradient of chemoattractant.

The anaphylatoxin C5a and the chemotactic fMLP peptide are both potent activators of actin polymerization in neutrophils and monocytes [53,54]. Studies concerning neutrophil supernatants [55–57] and involving fMLP-receptor stimulation [57,58], have provided evidence that WASP proteins and *de novo* Arp2/3-nucleation activity account for a great proportion of actin nucleation and assembly. However, the possibility that alternative, i.e. Arp2/3-independent, mechanisms could be involved in actin-nucleation activity is not precluded [57,59]. The convergence of the C5a- and fMLP-mediated signal transduction on a common pathway suggests that the same *de novo* Arp2/3-dependent-nucleation mechanism is triggered by both chemoattractants. WASP appears to be essential for the directed migration of cells, since patients with deficient WASPs have myeloid cells that display an altered ability to migrate up a gradient of chemoattractant [60–63]. The mechanism by which WASP is targeted to the leading edge is still unclear.

In the present study, we have shown that the cytosolic C-terminal part of C5aR interacts with C-terminal-truncated WASP fragments (fragments 1–321 and 1–420, referred to as WASP.321 and WASPΔVCA respectively) either in the yeast two-hybrid assay or in the pull-down and immunoprecipitation assays. This contrasts strikingly with the inability of full-length WASP to interact with the C-terminal region of C5aR in the yeast two-hybrid assay and the poor capacity of WASP to translocate to the plasma membrane and interact with C5aR in transfected cells. This difference is most likely due to the auto-inhibited 'closed conformation' of WASP. *In vitro* studies have shown that the binding of PtdIns(4,5)P_2 and the interaction of GTP-bound Cdc42 to the GBD domain of WASP release this regulatory mechanism and switch WASP to an activated 'open conformation' [34–36]. In the case of WASP.321 and WASPΔVCA, such a structural constraint does not exist owing to the lack of VCA domain. The C-terminal sequence of WASP.321, shortly downstream of the GBD domain (aa 280–321) appears to be important for binding to the C-terminal domain of C5aR in the two-hybrid assay. This region encompasses a fragment sequence (aa 288–310) that has been found essential for binding to the VCA domain [36]. In full-length WASP, this region might be unmasked following binding of the activated form of Cdc42 as suggested by the observation that the amount of WASP pulled-down with C5aR is markedly increased when WASP and C5aR are co-expressed with Cdc42V12 (see Figure 4B). This suggests that, *in vivo*, a C5a-mediated activation of the small GTPase Cdc42 might promote the transient association of WASP with C5aR.

A transient interaction between C5aR and WASP might target WASP to the plasma membrane enabling the migrating cells to polymerize actin and extend pseudopods where the highest level of signal transduction takes place. Such a mechanism is reminiscent of the molecular interaction features described in the context of invading intracellular bacteria. In the case of *Shigella*, IcsA, the bacterial surface protein that is responsible for the formation of actin comets and bacterial locomotion in the cytosol of living cells, directly binds to neuronal WASP (N-WASP), a homologue of WASP that is present in the cytosol of host eukaryotic cells [64,65]. Further association of Arp2/3 to the VCA domain of N-WASP is critical [65]. In the case of *Listeria*, the eukaryotic Arp2/3 complex is recruited directly to the bacterial surface protein, ActA, at a region which is similar to the VCA domain of the WASP proteins [66,67]. These examples, although different in their detailed architecture, indicate that actin-filament nucleation is spatially controlled by the ternary physical association between a moving surface, a VCA domain and the Arp2/3 complex. The subsequent polymerization process, which thus occurs just adjacent to the membrane, is thought to raise movement by driving membrane-protruding forces. Interestingly, using WASP-coated microspheres in cell extracts, WASP was shown to be able, like N-WASP for *Shigella* or ActA for *Listeria*, to drive actin-based locomotion in a Arp2/3-dependent manner [34,68]. In leucocytes, the close association of WASP with C5aR might account for the membrane protrusion during pseudopod formation and chemotaxis in a gradient of C5a.

However, in cells that extend pseudopods and move in a chemical gradient, agonist binding is required to activate the receptor-associated actin machinery. The interaction between WASP.321 and C5aR was actually modulated by the presence of C5a. Our results with the GST pull-down assay suggest that the WASP.321–C5aR interaction is, to some extent, inhibited by the ligand-induced phosphorylation of the receptor C-terminal tail. Based on the yeast two-hybrid assay results, the only serine residue whose phosphorylation status might act as an on/off switch on the association of the receptor with WASP.321 is Ser^{327}. It is not possible to determine whether WASP.321 is able to bind to occupied C5aR, since there is no evidence that the fraction of phosphorylated receptors that precipitated with WASP.321 still possess bound ligand, in particular after detergent extraction in pull-down experiments.

Taken together, the results show that WASP.321 and full-length WASP are able to bind to unoccupied C5aR. However, under our experimental conditions, a high level of receptor expression was required to observe this interaction, so that a significant amount of receptor might wobble between several conformations that could be trapped by WASP.321. Hence, solubilization in detergent or overexpression of C5aR in transfected cells may artificially favour the interaction with WASP.321 and full-length WASP, even in the absence of ligand. Although we were unable to detect an interaction between WASP and C5aR by confocal immunofluorescence, the ability of DSP to cross-link WASP and C5aR in a cell context suggests that the two molecules are in a very close vicinity. Because in chemoattractant-stimulated cells, the actin response is very fast and very transient, the interaction between WASP and C5aR is also expected to be transient. The observation that the phosphorylation of C5aR at position 327 markedly reduces the interaction with WASP.321 suggests that full-length WASP and C5aR could only interact before C5aR is phosphorylated and binds firmly to β-arrestins. These two regulatory steps have been shown to occur in less than 2 min [32,69]. In contrast with the ligand-independent interaction between WASP.321 and C5aR, the interaction between full-length WASP and C5aR is likely to require ligand binding and activation

of downstream signalling events, namely the activation of Cdc42 that is thought to release the auto-inhibited conformation of WASP [34–36]. Thus, by providing a nucleating functional VCA domain to the plasma membrane, such a short-lived interaction of WASP with the agonist-occupied, but not yet phosphorylated, receptor may be a key signalling intermediate in actin polymerization.

A stoichiometric ligand/receptor/WASP-dependence in the induction of actin nuclei could provide the chemoattractant receptors with a means of controlling the precise area at the cell surface where actin has to polymerize. This obviously raises the question whether or not the interaction with WASP could be a general feature of chemoattractant receptors. When the interaction between different WASP constructs and the C-terminal domain of two members of the fMLP receptor family [N-formyl peptide receptor ('FPR') and fMLP-related receptor 1 (FPRL1)] was tested with the yeast two-hybrid assay, no interaction could be detected (results not shown). However, one cannot exclude the possibility that the interaction of WASP with chemotactic receptors requires additional structural determinants borne by the receptor cytosolic loops. This hypothesis is supported by the observation that full-length WASP could be cross-linked to, and co-immunoprecipitated with, FPRL1 when cells were co-transfected with FPRL1, WASP and Cdc42V12 (results not shown). Although additional studies are required to allow a generalization to other chemotactic receptors, we believe that the present study provides a potentially exciting hypothesis for future work.

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