Phosphorylation of filamin A regulates chemokine receptor CCR2 recycling

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ABSTRACT

Proper endosomal trafficking of ligand-activated G-protein-coupled receptors (GPCRs) is essential to spatiotemporally tune their physiological responses. For the monocyte chemoattractant receptor 2 (CCR2B; one of two isoforms encoded by CCR2), endocytic recycling is important to sustain monocyte migration. Here, we analyze the role of FLNa in the trafficking of CCR2B in the endocytic pathway. In FLNa-knockdown cells, activated CCR2B accumulated in enlarged EEA-1-positive endosomes, which exhibited slow movement and fast fluorescence recovery, suggesting an imbalance between receptor entry and exit rates. Utilizing super-resolution microscopy, we observed that FLNa–GFP, CCR2B and β2-adrenergic receptor (β2AR) were present in actin-enriched endosomal microdomains. Depletion of FLNa decreased CCR2B association with these microdomains and concomitantly delayed CCR2B endosomal traffic, without apparently affecting the number of microdomains. Interestingly, CCR2B and β2AR signaling induced phosphorylation of FLNa at residue S2152, and this phosphorylation event was contributive to sustain receptor recycling. Thus, our data strongly suggest that CCR2B and β2AR signals to FLNa to stimulate its endocytosis and recycling to the plasma membrane.

KEY WORDS: GPCR, CCR2, Filamin A, Recycling

INTRODUCTION

A key aspect of the cellular responses mediated by G-protein-coupled receptors (GPCRs) is the spatiotemporal regulation of their signaling, which occurs at the level of ligand binding but also through control of the number of receptors on the cell surface. Therefore, explaining the control of receptor sorting into different degradative or recycling endocytic pathways is fundamental to understand GPCR function. Once internalized, receptors follow different fates inside the cell: some rapidly return to the plasma membrane from the sorting endosome, others travel to the perinuclear endosomes for slower recycling or are transported to lysosomes for degradation. Receptor sorting within endosome trafficking is facilitated by dynamic actin filaments, which are generated through WASH and the Arp 2/3 complex at the surface of endosomes (Derivery et al., 2009; Gomez and Billadeau, 2009) and specialized protein interactions. Some GPCRs, such as β2-adrenergic receptor (β2AR), have specific sequences that trap the receptor in endosomal actin-enriched subdomains for transport along the short recycling route – so called ‘sequence-dependent’ recycling (Cao et al., 1999; Pathak et al., 2010). The sequence-dependent pathways require PDZ-containing proteins and the retromer complex (Temkin et al., 2011). In those routes, the actin-enriched subdomains are thought to function as platforms that capture cargo proteins into retromer tubules, retrieving them from the default pathway.

The diversity of actin functions along the endocytic pathway is the result of proteins that precisely control the architecture of the actin networks and of the interacting partners among the endocytic cargo and the endocytic machinery. Some of the most prominent actin-cross-linking proteins in higher eukaryotes are the filamins (filamin A, B and C; FLNa, FLNb and FLNc, respectively) (Nakamura et al., 2011). FLNa is a dimeric protein with 24 immunoglobulin (Ig) repeats that cross-link rod-like actin filaments at right angles, creating three-dimensional networks (Hartwig et al., 1980). FLNa exists as an autoinhibitory form that involves Ig repeat 20 (Lad et al., 2007). In addition, FLNa binds to a myriad of cellular proteins of great functional diversity, acting as a scaffold and facilitating protein–protein interactions (Popowicz et al., 2006). Thereby, FLNa integrates the cellular architecture and signaling cascades that are essential for fetal development and cell locomotion. Not surprisingly, mutations in FLNa are linked to a large number of developmental diseases (Savoy and Ghosh, 2013). Among its partners are several GPCRs (Awata et al., 2001; Kim et al., 2005; Onoprishvili et al., 2003; Yu et al., 2008), including several chemokine receptors: CCR2B (one of two alternative splice forms encoded by CCR2) (Minsaas et al., 2010; Planagumà et al., 2012), CCR5 and CXCR4 (Gómez-Moutón et al., 2015; Jiménez-Baranda et al., 2007). The exact role of FLNa binding to chemokine receptors is not well understood yet, but much work indicates its function in endocytic traffic and receptor internalization (Cho et al., 2007; Kim et al., 2005; Onoprishvili et al., 2003; Seek et al., 2003). Moreover, FLNa binds to β-arrestin-2, a well-defined GPCR adaptor for clathrin-mediated internalization (Kim et al., 2005; Scott et al., 2006). Recently, FLNa has been suggested to cooperate with binding of β-arrestin-2 to the CXCR4 receptor carrying mutations in arginine residue 334 that are present in WHIM syndrome, which could have implications in WHIM-mutant CXCR4 signaling (Gómez-Moutón et al., 2015).

We have previously shown that FLNa, through predominantly Ig repeats 19–23, binds to the C-terminal tail of the monocyte chemoattractant receptor 2 (CCR2, isoform B) (Minsaas et al., 2010). CCR2 and its ligand, the monocyte chemoattractant protein 1
CCR2B trafficking through the endocytic pathway, the other than those previously reported. To analyze the role of FLNa in suggests that FLNa plays roles in CCR2B endosomal trafficking, receptor recycling upon FLNa depletion (Minsaas et al., 2010). This compared with the isogenic cell line (Minsaas et al., 2010). Initial human melanoma cell line M2 (lacking expression of FLNa) to be more peripherally distributed upon ligand stimulation in the activated receptor into clathrin-coated pits (Minsaas et al., 2010). 

We noticed that endosomal structures containing CCR2B appeared enlarged of the CCR2B-loaded organelles in cells that had been loaded endosomes by live confocal imaging revealed an apparent enlargement of the CCR2B-positive endosomes in the absence of FLNa over at least 15 minutes. Quantification of fluorescence indicated that knockdown of FLNa expression increased the colocalization of CCR2B with EEA-1 endosomes 1.6-fold ($P<0.05$, two way ANOVA) at 30 min, compared to controls (Fig. 1D). This implied that, in the absence of FLNa, the EEA-1-labeled endosomes kept their capacity to receive incoming vesicles from the plasma membrane for at least 30 min after addition of CCL2 and that the exit of CCR2 from early endosomes was delayed.

FLNa contributes to early endosomal motility
As the receptor accumulates in enlarged early endosomes in the absence of FLNa, we studied the effect of FLNa depletion on the dynamic behavior of CCR2B-loaded endosomes using high-speed [20 frames per second (fps)] imaging. Analysis of immunolabeled CCR2B-positive endosome trajectories that lasted more than 1 min showed a reduction in endosomal velocity [25±5% (mean±s.e.m.) slower than that of controls] and distance traveled [30±5.5% (mean±s.e.m.) shorter than that in controls] in shFLNa cells (Fig. 2A). Significant differences were observed within 15 and 30 min after addition of CCL2, which were independent of the size of the endocytic structures. These experiments allowed us to observe long-range endosome trajectories directed towards the perinuclear region in control cells, whereas in shFLNa cells, CCR2B endosomes moved in random directions with their trajectories remaining close to the origin (Fig. 2B).

To analyze the balance between CCR2B entry and exit rates in and out of sorting endosomes, fluorescent resonance after photo-bleaching (FRAP) experiments were conducted in living cells expressing Rab4A–YFP, loaded with Alexa-Fluor-647-conjugated antibodies against CCR2B. Endosomes with similar levels of CCR2B–Alexa-Fluor-647 fluorescence and size were selected, and one whole endosome was bleached in its entirety. After FRAP, only 11±1.3% (mean±s.e.m.) of the receptor signal on Rab4A-containing endosomes was recovered after 2.5 min in control cells (Fig. 2C, upper panels; Movie 1). The recovery of the CCR2B signal was faster in the shFLNa cells [18±1.8% (mean±s.e.m.) after 2.5 min (Fig. 2D), compared to controls and to bleached Rab4A–YFP, which showed no differences (Fig. S2; Movie 2). This was despite FLNa depletion hindering the internalization of CCR2B and the motility of the primary endocytic vesicles. Taken together, the data strongly suggest that the exit of CCR2B from sorting endosomes was impaired in the absence of FLNa.

FLNa facilitates CCR2B and β2AR loading in actin rich subdomains
To study which route CCR2B follows back to the plasma membrane, we analyzed the extent of its colocalization with Rab4A and compared it with that of other well-known receptors [transferrin (Tfn) receptor, β2AR, PAR1 (also known as F2R) and ETB (also known as EDNRB)] (Fig. 3A,B). CCL2-stimulated CCR2B, as well as the other receptors, accumulated in microdomains along the Rab4A-decorated endosome membrane. Utilizing line fluorescence correlation (Fig. 3A,B), we found that Tfn was present in most Rab4A-labeled microdomains; consistent with previous reports showing the Tfn receptor can follow both the short- and the long-loop recycling routes (Mayle et al., 2012). Other GPCRs (PAR1 and Etb), which are sorted to lysosomes (Shapiro and Coughlin, 1998; Terada et al., 2014), hardly correlated with Rab4A microdomains (Fig. 3B; Fig. S3). In contrast, internalized CCR2B coincided with about 20% of the Rab4A-enriched microdomains, similar to β2AR, which mainly follows the short recycling loop from the sorting endosome to the plasma membrane.

RESULTS

CCR2B accumulates in enlarged early endosomes in FLNa-depleted cells
CCR2B directly interacts with the Rod 1 domain of FLNa, within Ig repeat 19, through the C-terminal intracellular domain of the receptor. This interaction facilitates internalization of the ligand-activated receptor into clathrin-coated pits (Minsaas et al., 2010). We noticed that endosomal structures containing CCR2B appeared to be more peripherally distributed upon ligand stimulation in the human melanoma cell line M2 (lacking expression of FLNa) compared with the isogenic cell line (Minsaas et al., 2010). Initial experiments published previously had also indicated a defect in receptor recycling upon FLNa depletion (Minsaas et al., 2010). This suggests that FLNa plays roles in CCR2B endosomal trafficking, other than those previously reported. To analyze the role of FLNa in CCR2B trafficking through the endocytic pathway, the internalization of Flag–CCR2B was followed with live confocal microscopy in HeLa cells that had been depleted of FLNa by utilizing short hairpin (sh)RNAs (Fig. 1A; Fig. S1A). The results were consistent with previous data and showed CCR2B in peripheral endosomal structures in the absence of FLNa, 30 min after addition of CCL2 (Fig. S1B). Close observation of receptor-loaded endosomes by live confocal imaging revealed an apparent enlargement of the CCR2B-loaded organelles in cells that had been treated with two different shRNAs against FLNa (shFLNa28 and shFLNa29) (Fig. 1A; Fig. S1B).

To study the nature of the enlarged endosomal particles, HeLa cells that expressed CCR2B were stimulated with CCL2 at different times, fixed and co-labeled with antibodies against Flag and EEA-1 (Fig. 1C; see Fig. S1C for lower magnification). Some internalized CCR2B particles colocalized with EEA-1 in the presence or absence of FLNa 15 min after CCL2 addition, but the localization of internalized CCR2B with EEA-1 became more apparent in shFLNa cells [11±1.3% (mean±s.e.m.) of the receptor signal on Rab4A-containing endosomes was recovered after 2.5 min in control cells (Fig. 2C, upper panels; Movie 1). The recovery of the CCR2B signal was faster in the shFLNa cells [18±1.8% (mean±s.e.m.) after 2.5 min (Fig. 2D), compared to controls and to bleached Rab4A–YFP, which showed no differences (Fig. S2; Movie 2). This was despite FLNa depletion hindering the internalization of CCR2B and the motility of the primary endocytic vesicles. Taken together, the data strongly suggest that the exit of CCR2B from sorting endosomes was impaired in the absence of FLNa.
In fact, CCR2B (Flag–CCR2B) and β2AR (HA–β2AR) often colocalized or were found in close apposition on endosomal microdomains and moved together along the endosomal membrane (Fig. 3C,D), indicating that they follow the same trafficking pathway. As previously described (Temkin et al., 2011), β2AR microdomains were positive for coronin2–GFP, which marks the actin-enriched structures that trap cargo destined for the short-loop recycling pathway from sorting endosomes to the plasma membrane (Fig. 3E, lower panel). Consistent with the view that CCR2B followed the same route as coronin2, CCR2B-labeled microdomains also localized with coronin2–GFP (Fig. 3E, see also line profile).

To investigate if FLNa might be directly involved in the endosomal trafficking of CCR2, super-resolution microscopy (STED) was utilized to determine its presence on endosomes. The fluorescence signal of a functional FLNa–GFP construct (Planagumà et al., 2012) was most apparent at the cortex of HeLa cells (Fig. 4A). However, taking a sagittal plane of the cell, FLNa–GFP could be detected at discrete dynamic subdomains on the surface of vacuolar structures (Fig. 4A, lower panel). Interestingly, FLNa–DsRed colocalized with coronin2–GFP dots on the vacuolar membranes, indicating that FLNa might directly participate in the function and regulation of the actin-enriched microdomains in trapping cargo for the retromer pathway to the plasma membrane (Fig. 4B). To confirm so, we investigated whether some of these FLNa–GFP microdomains coincided with internalized fluorescence-labeled CCR2B. CCR2B was present in Rab4A–Cherry-labeled sorting endosomes in accumulations, which transiently coincided with FLNa–GFP microdomains (Fig. 4D). It was also possible to observe FLNa together with CCR2B on domains in Rab5A–Cherry-labeled endosomes (Fig. 4D; Movie 4). However, little coincidence was observed in Rab11–Cherry-labeled endosomes (Fig. 4D; Movie 5). These results indicate that FLNa and CCR2B colocalized on the endosomal actin and coronin2-enriched early endosomal microdomains, similar to internalized HA–β2AR (Fig. 4E, see also Fig. 5).

We next asked if FLNa is important to maintain the actin-enriched endosomal subdomains. Depletion of FLNa did not alter either the number or the intensity of the coronin2–GFP patches on the endosomes (Fig. 5A,B), suggesting that FLNa is not essential to generate or maintain the structure of coronin2–actin patches on endosomes. Strikingly though, we observed that depletion of FLNa clearly decreased the association of fluorescence-labeled FLNa knockdown promotes accumulation of CCR2B at early endosomes. (A) Confocal micrographs of live HeLa cells that had been transfected with shRNAs against FLNa (shFLNa28 and shFLNa29, also referred to as sh28 and sh29, respectively) or a scrambled control (shScr) and plated in glass chambers. The cells also expressed Flag–CCR2B, were surface labeled with anti-Flag and Alexa-Fluor-488-conjugated anti-mouse antibodies. Images were captured 60 min after CCL2 addition in a sealed chamber under 5% CO2 at 37°C. See Fig. S1C for images with low magnification. (B) Box plots showing the endosome diameter measured using the line tool of the software Las A1 (Leica microsystems). Lines represent the median, and boxes show the 25th and 75th percentiles of the endosome diameters in sh28, sh29 and shScr cells. Whiskers indicate s.d. A minimum of eight cells in three separate experiments were quantified with 30 endosomes per cell, approximately. Data were analyzed using one way ANOVA test (**P<0.01). (C) Confocal micrographs of HeLa cells expressing shFLNa28 and shScr along with CCR2B, were incubated with 20 nM CCL2 for 15 min at 37°C, fixed and immunostained with anti-EEA-1 antibodies followed by Alexa-Fluor-488-conjugated and M1 anti-Flag antibodies, and then with an Alexa-Fluor-568-conjugated secondary antibody, washed and mounted with ProLong. Magnified images corresponding to small boxes are shown in the insets. Scale bars: 10 μm. See Fig. S1C for a wider-field image. (D) Quantification of the EEA-1 that colocalized with CCR2B in cells treated as described in C, 15 and 30 min after addition of 20 nM CCL2. Significance was determined using one way ANOVA testing of three independent experiments. A significant difference was not found between 15 and 30 min for each shRNA-treated cell line, and there was a significant difference between shScr and shFLNa at each time point (*P<0.05). Imaging was performed with a Leica TCS SP5 using a 63×1.3 NA oil immersion lens. Scale bars: 10 μm.
CCR2B with coronin2 on Rab4A-positive endosomal membranes (Fig. 5A,C). Likewise, depletion of FLNa also diminished β2AR-receptor-loading in coronin2-positive domains (Fig. 5C). These results suggest that FLNa might be required for efficient loading of some GPCRs onto the actin-enriched sorting endosome microdomains.

FLNa is required for efficient CCR2B and β2AR recycling

If FLNa contributes to the concentration of certain GPCRs into domains for subsequent loading of transport intermediates from the sorting endosomes to the plasma membrane, its absence should alter receptor endocytic transport and recycling. We analyzed the recycling kinetics of CCR2B and β2AR in FLNa-depleted cells by using fluorescence microscopy to measure the return of the internalized receptor back to plasma membrane. Consistent with our hypothesis, knocking down FLNa produced a significant delay in the kinetics of the retrograde trafficking of the receptors to the plasma membrane (Fig. 5A,C). Likewise, depletion of FLNa also diminished β2AR-receptor-loading in coronin2-positive domains (Fig. 5C). These results suggest that FLNa might be required for efficient loading of some GPCRs onto the actin-enriched sorting endosome microdomains.

Our data strongly indicate that FLNa acts as a trafficking adaptor rather than as a structural protein during endosomal trafficking to the plasma membrane. To further investigate this matter, we next asked whether the FLNa regulation of CCR2B recycling involved the FLNa actin-binding domain (ABD), which forms the orthogonal actin networks (Janmey et al., 1990). Strikingly and consistent with our hypothesis, the mouse mutant construct FLNa-ABD–DsRed, with the actin-binding domain deleted (Muriel et al., 2011), resulted in a similar level of receptor recycling to that with wild-type mouse FLNa (Fig. 6E; Fig. S3A). Confocal micrographs of mouse FLNa-ABD–DsRed denoted a prominent cytoplasmic distribution of the mutant, which hindered its visibility on vacuolar structures, which nevertheless could still be observed in some cells (Fig. S3A). These results suggest that the predominant actin cross-linking activity of FLNa might not be an important function of the protein in CCR2B recycling; however, we cannot completely rule out the possibility that the second actin-binding domain of FLNa that has been reported previously (Nakamura et al., 2007) is sufficient for receptor recycling. These results agree with our previous observations that FLNa does not affect actin–coronin2 patches but might facilitate loading of certain receptors into these microdomains.

FLNa is phosphorylated at multiple sites by several protein kinases (Nakamura et al., 2011). Among these, phosphorylation at residue S2152 (Muriel et al., 2011; Tigges et al., 2003; Travis et al., 2004) has a clear impact on FLNa function. To study whether FLNa is phosphorylated at multiple sites by several protein kinases (Nakamura et al., 2011). Among these, phosphorylation at residue S2152 (Muriel et al., 2011; Tigges et al., 2003; Travis et al., 2004) has a clear impact on FLNa function. To study whether FLNa
phosphorylation is required to sustain CCR2B recycling, we analyzed the capacity of FLNa-S2152 mutants to revert receptor recycling defects in FLNa-knockdown cells by using flow cytometry to analyze shFLNa cells (Fig. 6E). Western blot analysis demonstrated that all constructs and mutants (regardless of whether they were tagged with GFP or DsRed) were expressed at similar levels (Fig. 6E,F, upper panels). We observed that the phosphorylation mimetic FLNa mutant, S2152E, supported receptor recycling similar to wild-type FLNa. However, the non-phosphorylatable FLNa-S2152A mutant only partially recovered CCR2B recycling. Consistent with this, the phospho-mimetic mutant (FLNa-S2152E–DsRed or FLNa-S2152E–GFP) was present on endosomal membranes (Fig. 6F; Fig. S3A,B), whereas the GFP- or DsRed-tagged FLNa-S1252A mutant could hardly be detected on these organelles (Fig. 6F; Fig S3A,B). This was despite the fact that both mutants were present at the cellular cortex.
Phosphorylation of FLNa at residue S2152 regulates its localization on endosomal structures. CCR2 and β2AR signaling induces phosphorylation of FLNa at S2152

We also investigated whether the activation of CCR2B could itself induce FLNa phosphorylation as a mechanism to control its own trafficking. For this, we utilized a specific antibody against FLNa phosphorylated at S2152. The specificity of the antibody was validated by immunoblot analysis of cells expressing the FLNa-S2152A mutant in shFLNa-transfected HEK293 cells (Fig. 7A). Only the wild-type form of FLNa but not the FLNa-S2152A mutant was detected by the antibody. Notice that only the long form of FLNa–GFP that contained the phosphorylation site was recognized by the antibody (Fig. 7A, see arrows). Treatment with CCL2 induced a small but significant increase in FLNa phosphorylation, 5 and 15 min after CCL2 stimulation (Fig. 7B), which paralleled the activation of ERK by CCL2 (Fig. S4A–C). CCL2 did not induce any increase in phosphorylation of the FLNa-S2152A mutant that had been expressed in shFLNa HEK293 cells (Fig. 7A, lower panel). Different kinases have been implicated in the phosphorylation of FLNa, including several isoforms of PKC and PKA, which are also downstream effectors of GPCR signaling. Treatment with different protein kinase inhibitors targeting PKCs [GF10903, Gö6976 and bisindolylmaleimide II] (Fig. 7C–E) consistently produced a significant reduction of FLNa phosphorylation at S2152 upon CCL2 treatment. The reduction of phosphorylation of FLNa at S2152 in CCL2-stimulated cells upon treatment with GF10903 was dose dependent (Fig. 7D). In contrast, no inhibition of FLNa phosphorylation at S2152 was observed in the presence of the PKA inhibitor H-89, even at relatively high doses. β2AR stimulation also led to FLNa phosphorylation on S2152 after 5 and 15 min of isoproterenol treatment (Fig. 7F,G). Interestingly, however, phosphorylation of FLNa at S2152 induced by β2AR was not reduced in the presence of GF10903 but on the contrary, it was reduced in the presence of H-89. Again, isoproterenol stimulation did not result in any phosphorylation of the FLNa-S2152A mutant (Fig. 7F). Therefore, we can conclude that both receptors, CCR2B and β2AR, can induce phosphorylation of FLNa at S2152 upon ligand stimulation, most probably through different downstream pathways.

DISCUSSION

Our work demonstrates a very specific role of FLNa in promoting the recycling of CCR2B from sorting endosomes to the plasma membrane. We showed that FLNa and internalized CCR2B colocalized in dynamic actin- and coronin2-enriched endosomal subdomains, which are known to concentrate cargo for the Rab4A- and retromer-dependent pathway to the plasma membrane (Puthenveedu et al., 2010; Temkin et al., 2011), which is also used by other GPCRs like β2AR and AVPR2 (V2R). Interestingly, even though filamins have a prominent role as actin cross-linkers, we also

(Fig S3B,C). This suggests that phosphorylation of FLNa at residue S2152 regulates its localization on endosomal structures.

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In addition to a direct role of FLNa in CCR2B and β2AR recycling, our data demonstrated that FLNa depletion greatly reduced the long-term motility of the sorting endosomes and increased their size. Depletion of WASH (Gomez et al., 2012), or the Arp2/3 complex (Derivery et al., 2012), also causes early endosome enlargement. In these conditions, endosomes present enlarged segregated subdomains with collapsed endosomal and lysosomal networks. They do not exhibit elongated tubulations and are devoid of filamentous actin. Although FLNa depletion increased the endosome size, it did not seem to alter the actin-rich endosome domains, as the number and size of the coronin2 patches remained the same and no collapse of endosomal networks was observed, which suggest that role of FLNa differs from that seen in WASH and Arp2/3 studies. Whether the endosomal phenotypes observed by FLNa depletion are a cause or a consequence of the recycling defect, or are unrelated, is a complex question to address in the future. Thus, a defect in the recycling of certain cargo might prevent maturation of endosomes and their proper attachment to microtubules for long-range movement. Reciprocally, the enlargement of endosomes might be caused not only by the accumulation of certain cargo unable to exit the organelle but also by maintenance of the endosome capacity to accept vesicles from the plasma membrane, provided that they do not move away from the cortex. Alternatively, or additionally, depletion of FLNa might directly alter tubulin-based motility, or the architecture of microtubules (Lynch et al., 2011), and as a consequence, indirectly affect endosomal motility, size and distribution (Bayer et al., 1998) without having a dramatic effect on the actin cytoskeleton structure (Baldassarre et al., 2009). In any case, a general alteration of the endosome motility or distribution is unlikely to be the primary cause of the CCR2B recycling defects because the kinetics of Tfn transport from the endosomes to plasma membrane appeared to be unaltered in FLNa-depleted cells (Fig. 6C,D; Minsaas et al., 2010), as did its uptake (Muriel et al., 2011). We favor the hypothesis that FLNa acts as a scaffold that cooperates to load the cargo into actin-enriched domains. Concomitantly, FLNa would be needed to maintain the distribution and motility of endosomes by interacting with actin and/or microtubule networks. This hypothesis agrees with our previous results showing that upon activation, CCR2B-loaded vesicles were present in a linear distribution with directional movement, co-aligned with actin fibers and FLNa (Minsaas et al., 2010; Planagumà et al., 2012). It is interesting that FLNa regulates the linear distribution of caveolae along stress fibers (Muriel et al., 2011) and their translocation from the plasma membrane to perinuclear Rab11 structures.

FLNa is regulated by phosphorylation at S2152 by many protein kinases, and this phosphorylation has a direct effect on its function in regulating a variety of cytoskeleton-related events (Hastie et al., 1997; Muriel et al., 2011; Travis et al., 2004; Zhang et al., 2012). Here, we show that phosphorylation of the same residue is necessary for efficient recycling of CCR2B and β2AR. Together with the fact that the S2152A mutant of FLNa was not present in endosomes, our data suggest that phosphorylation of FLNa at S2152 facilitates the accumulation of this protein on endosomal microdomains (see model in Fig. 7I). These results agree with previous data where Myc-tagged FLNa-S2152A localizes with actin fibers at the cell surface, whereas phosphorylation of FLNa directs it towards the cytoplasm of CHP100 cells (Zhang et al., 2012). We also showed here that CCR2B and β2AR signaling can trigger phosphorylation of FLNa at S2152. Interestingly, our data also suggests that phosphorylation of FLNa at S2152 by these GPCRs follows different pathways and uses different kinases because CCR2-
induced phosphorylation of FLNa at S2152 was reduced by PKC inhibitors but not by PKA inhibitors. Reciprocally, β2AR-induced phosphorylation relied more on a PKA-dependent pathway. The data are consistent with the different signaling cascades ignited by phosphorylation relied more on a PKA-dependent pathway. The present data, it reinforces the concept that ligand-dependent phosphorylation of FLNa by GPCRs becomes an important pathway for FLNa activation by promoting the phosphorylation of S2152. Overall, we favor the hypothesis that a signaling response leading to activation of different second messenger protein kinases to phosphorylate FLNa, which in turn will induce its detachment from the actin structures at the cell cortex and facilitate its localization in highly dynamic actin-enriched endosomal microdomains (Fig. 7I). This mechanism probably operates for other chemokine receptors and other GPCRs.

MATERIALS AND METHODS

Constructs and reagents

Plasmids encoding FLNa–EGFP, Flag–CCR2B (Planagumà et al., 2012), FLNa-S2152A–DsRed and FLNa-S2152E–DsRed (Muriel et al., 2011) were derived from pcDNA3.1-FLNa-EGFP. Human Rab4A–YFP and human Rab5A–GFP were provided by Peter van der Sluijs (University
Fig. 7. Phosphorylation of FLNa at S2152 by CCR2 signaling. (A) Validation of the antibody against phosphorylated FLNa (pFLNa). Upper panels, immunoblots for FLNa phosphorylated at S2152 (pFLNa), which were then stripped and reprobed for total FLNa in protein extracts of shFLNa HEK293 cells that expressed FLNa (wt) or FLNa-S2152A (SA) under control or starving conditions (sv). Lower panels, immunoblots against FLNa phosphorylated at S2152, which were then stripped and reprobed for total FLNa in protein extracts of shFLNa HELa cells expressing Flag–CCR2B together with FLNa or FLNa-S2152A and stimulated with 20 nM CCL2 for 15 min. (B) Immunoblots of HELa cells that expressed CCR2B were stimulated with 20 nM CCL2 for 5 and 15 min. Lysates were subjected to immunoblotting with antibodies against pFLNa and phosphorylated ERK (pERK), and reprobed with anti-FLNa and anti-ERK 1 and -ERK2 antibodies (see Fig. S4 for CCL2-induced pERK assays). β-tub, β-tubulin. (C) HELa cells were pretreated with 1 µM GF10903 (GF) or DMSO for 1 h and stimulated with 20 nM CCL2 for 5 and 15 min. Graphs (B,C) show the amount of pFLNa (quantified in the linear range with an Odyssey imager) normalized to the corresponding FLNa signal from three different experiments, expressed as the average fold-increase ± s.e.m. of FLNa phosphorylation relative to that at time 0 (right panels) (* P < 0.05, ** P < 0.01, *** P < 0.001; two-tailed t-test). (D) HELa cells that had been transfected with CCR2B and pretreated with increasing concentrations of H-89 (0.1, 0.5, 5 and 20 µM) and GF10903 (GF, 0.02, 0.1, 0.5, 1 µM) were stimulated with 20 nM CCL2 for 15 min. (E) Cells as described in D were pretreated with C66796 (2 µM) or with bisindolylmaleimide II (Bl, 2 µM) or DMSO for 1 h. (F) HELa cells that had been transfected with Flag–j2AR (B2AR) and pretreated with 1 µM GF10903 and 20 µM H-89 were stimulated with 10 µM isoproterenol (Iso) for the times indicated. (G) A graph showing the quantification of pFLNa after stimulation for 15 min with 10 µM isoproterenol or in cells that had been left unstimulated. (H) shFLNa HEK293 cells that expressed FLNa (wt) or FLNa-S2152A (SA) and had been transfected with Flag–CCR2B and Flag–j2AR (B2AR) were stimulated with 20 nM CCL2 and 10 µM isoproterenol, respectively. (I) A model of homeostatic regulation of CCR2B recycling. Phosphorylation of S2152 of FLNa in response to CCL2 facilitates the entry of CCR2B into actin-positive tubules and its recycling. Tfr, transferrin receptor; pSerine2152, FLNa phosphorylated at S2152.
anti-phospho ERK1/2 and anti-ERK1/2 (p44/42 MAPK 137F5 #4695; Cell Signaling; dilution 1/1000), and anti-β-tubulin (#T6199, Sigma; 1/1000 dilution) antibodies. Alexa-Fluor-647- (#A21245, lot#651740; #A21236, lot# 716822) and Alexa-Fluor-488-conjugated (anti-mouse #A21202, lot #536050) secondary antibodies were from Invitrogen (all used at 1:1000 dilution). CF488A- and CF568-labeled M1 anti-Flag antibodies (Sigma-Aldrich) were obtained with Mix-n-Stain™ CF™ Antibody Labeling Kit (MX488AS100-1KT, Sigma-Aldrich) following the manufacturer’s instructions. A mouse anti-EA-1 antibody (#610457, BD Transduction Laboratories) was detected by an anti-rabbit secondary antibody after treating first the antibody with Affinity Pure Fab Fragment Rabbit Anti-Mouse IgG (H+L) according to the manufacturer’s instructions (#315-007-003, Jackson ImmunoResearch). For western blot infrared analysis (Odyssey, LI-COR), IRDye680- and IRDye800-conjugated anti-rabbit and anti-mouse secondary antibodies were used (800CW anti-rabbit #926-32311; 800CW anti-mouse #926-32210, lot #C1102603 from Odyssey; anti-rabbit IRDye 680RD #926-68073, lot# C30724-04 from Odyssey, all used at 1:20000 dilution). CCL2 and human MCP-1 were from PeproTech (Rocky Hill, NJ); [Arg5]-vasopressin acetate salt and endothelin 1 were purchased from Sigma-Aldrich and isoproterenol was obtained from Calbiochem.

**Cell treatments and western blotting**

HeLa cells were obtained from American Type Culture Collection and always kept under low passage number from the initial clone. HEK293 (293-EBNA) were bought from Life Technologies and cultured in the presence of G418. All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere under 5% CO2. Cells were regularly tested for mycoplasma contamination. All cells were grown and transfected utilizing FuGene® 6 (Roche) in all experiments. Cells were treated with 20 nM of CCL2 or 10 μM isoproterenol in culture medium for 5 and 15 min prior to analysis. The following concentrations of ligands were also used: 1 μM vasopressin and 10 nM endothelin. For protein kinase inhibition, cells were treated with varying concentrations of GF109203X (inhibitor of PKCα, β, βII and γ subtypes) (ENZO Life Sciences), H-89 (general inhibitor of protein kinase A), 2 µM Gö6976 (general PKC inhibitor) (Calbiochem) and 500 µM bisindolylmaleimide II (general PKC inhibitor) (Calbiochem) for 60 min. Utilizing the MISSION shRNA human system (Sigma-Aldrich), FLNa shRNA (shRNA) against human FLNa (TRCN0000062528 and TRCN0000062529) was used in HeLa and HEK293 cells. Virus production was performed as described previously (Masià-Balagué et al., 2015). Cells were solubilized in N-dodecyl-D-maltoside buffer (20 mM Tri-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1% N-D-maltoside, 10 mM NaF, 1 mM NaVO4 and protease inhibitors), as described previously (Masià-Balagué et al., 2015). The same amount of protein was loaded for each condition (measured by BioRad protein assay). Western blots were visualized and quantified by performing infrared detection (Odyssey System) in the linear range.

**Cell imaging**

For live imaging, HeLa or HEK293 cells that had been transiently transfected with FLNa-GFP, FLNa-GFP-S2152A, -S2152E, Rab4A-Cherry, Rab5-Cherry, Rab11-Cherry, actin–RFP and/or coronin2–GFP and Flag-tagged CCR2B receptors were plated on glass chambers and imaged at addition of anti-Flag antibody for 15 min at 4°C followed by an incubation with anti-mouse Alexa-Fluor-647-conjugated antibody (surface labeled) for 15 min at 4°C before addition of ligand at 37°C. HEK293 cells were also transfected with plasmids encoding Flag-tagged receptors: β2AR, Etb, V2R or PAR1. HA–β2AR was transiently transfected into HEK293 cells with Flag–CCR2B. Confocal imaging was performed with a Leica TCS SP5 (Leica Microsystems, Mannheim, Germany) by using a 63×1.3 NA oil immersion lens in a sealed chamber with 5% CO2 at 37°C. Movies were taken between 5 and 30 min after agonist addition. Each frame corresponds to 600 ms. For high-resolution fluorescence imaging, a STED microscope (Leica Microsystems) was utilized on live HeLa cells that expressed FLNa-GFP. For fixed cell imaging, HEK293 cells that had been transiently transfected with Flag–CCR2B and plated on glass chambers were treated with CCL2 (20 nM) for 15 or 30 min at 37°C before fixation, and processed for immunofluorescent staining (Planaguma et al., 2012). After fixation and permeabilization, cells were incubated with anti-Flag and anti-EA-1 antibodies, followed by incubation with the corresponding Alexa-Fluor-conjugated secondary antibodies (as stated in each figure legend).

**Fluorescence recovery after photobleaching**

Live-cell confocal imaging was performed on transiently transfected Rab4A–Cherry and/or Flag–CCR2B (surface labeled) control (scrambled shRNA) and shFLNa HEK293 cells at 37°C. Images were recorded as 16-bit sequences with a pinhole of 246 μm, giving a z-slice of 2 μm, and acquired at 3-s intervals. A 100-nW LASOS argon laser, set to 25% intensity, was used to photobleach a Rab4A endosome, and recovery was monitored for 26 cycles (10 s) with reduced laser power. A 488-nm line was used to follow the Rab4A-positive endosome and a 633-nm line was used to bleach the entire CCR2B endosome (4 μm2). Monitoring of receptor fluorescence recovery was done for 2.5 min. The mobile fraction (MF) was estimated as MF=(F1–F0)/(F0–F1)×100, where F1 is the final intensity upon recovery over the period of the experiment, F0 is the intensity before bleaching, and F0 is the intensity immediately after bleaching. Data were analyzed for 50 endosomes under each condition and were acquired on three different days. The background was subtracted and corrected by measuring the fluorescence decay of a non-bleached area in the same cell and normalized to the Rab4A signal (Rabut and Ellenberg, 2005).

**Recycling assays**

Quantification of receptor recycling was performed as previously described (Patel et al., 2011) using confocal microscopy. Cells that expressed Flag–CCR2B or Flag–β2AR were incubated with anti-Flag antibody at 4°C for 15 min, and endocytosis was initiated by addition of CCL2 or isoproterenol for 15 min at 37°C. Cells were acid-washed to eliminate traces of the antibody bound to the cell surface receptor and allowed to recover with DMEM for various time periods, washed with ice-cold PBS-Ca2+, fixed and incubated with Alexa-Fluor-647-conjugated antibody without permeabilization. Fluorescence intensities were averaged over individual cells and analyzed using the Fiji distribution of ImageJ. In each experiment, a minimum of four replicates were analyzed for each condition. The percentage of antibody that was recycled to plasma membrane was calculated as 1 minus the internal fluorescence values and was normalized against values at time 0 (post agonist) (results in Fig. 6A,B).

For the Tfn flow-cytometry-based recycling assay, HeLa cells were incubated with Alexa-Fluor-568-labeled Tfn at 0.02 mg/ml for 2 or 60 min at 37°C to allow internalization. At 2 min, only early endosomes are loaded with Tfn, and the kinetics of short-loop recycling can be measured. At 60 min, mainly recycling endosomes are loaded with Tfn and, therefore, traffic from the recycling endosomes is mostly measured. After acid stripping, to eliminate surface-bound fluorescent Tfn, the internalized fraction was chased at 37°C with 2 mg/ml of unlabeled Tfn for the indicated times. At each time point, cells were acid-washed, washed with ice-cold PBS and immediately detached using trypsin. Fluorescence intensity profiles of cell populations (10,000 cells per sample) were measured using a FacsAria I SORP (Becton Dickinson, San Jose, CA). Internal Tfn was normalized for the time 0 after the first stripping. The percentage of Tfn recycled was calculated as 1 minus the internal fluorescence values.

Analysis of CCR2B recycling was performed in HEK293 shFLNa cells that had been transiently expressing mouse FLNa-DsRed to rescue the recycling delay, and also cells expressing FLNa-ABD–DsRed, FLNa-S2152A–DsRed and FLNa-S2152E–DsRed (Fig. 6E). Conjugation of
anti-Flag M1 antibodies with CF488 was performed following the manufacturer’s instructions (Sigma-Aldrich). Conjugated CF488A–M1 anti-Flag antibody was incubated with cells for 15 min at 4°C prior to addition of 20 nM CCL2 and incubation for 15 min at 37°C to allow internalization. Then, cells were acid-washed and allowed to recycle back the receptor to the plasma membrane for the indicated times. Cells were again acid-washed, to exclusively eliminate the antibodies attached to the receptors reaching the plasma membrane, and trypsinized before analysis using a FacsAria I SORP instrument (Becton Dickinson). Green fluorescence intensity profiles were measured only in cell populations that expressed DsRed (5000 cells per sample). The fluorescence intensity measured is then a reflection of internalized receptor that remained intracellularly, which decreased with time upon chase.

Statistical analysis
Data are presented as mean values or as fold induction ± s.e.m. as indicated in each figure legend. The sum of all the individual stimulatory effects were compared to that of the combined effects by using the unpaired t-test with two-tailed P-values, or one-way ANOVA (Fig. 1B) or two-way ANOVA (Fig. 1D) with GraphPad Prism. Differences were considered statistically significant when *P<0.05, **P<0.01 and ***P<0.001.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.P., M.G., I.I. and A.M.A. conceived the research and designed the experiments; M.P., G.G., I.I., M.A.-C., J.P., O.M. performed experiments and analyzed data; M.P. and J.P. performed immunofluorescence experiments; M.D.P. provided reagents and designed experiments; M.P., M.G. and A.M.A. wrote the manuscript; and all authors read, added comments, and approved manuscript.

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Supplementary information
Supplementary information available online at http://jcs.biologists.orglookup doi:10.1242/jcs.193821.supplemental

References


**Fig.S1 . Analysis of shRNA against FLNa in HeLa cells.** (A) HeLa cell lysates of shScr or shFLNa28 and shFLNa29 analyzed by Western blot using anti-FLNa antibody and anti-βtubulin. (B) Flag-CCR2B-CCL2 stimulated appears in bigger vesicular structures in shFLNa than shScr HeLa cells. Confocal micrographs of shScr and shFLNA28 HeLa cells transiently transfected with pcDNA3-Flag-CCR2B. Cells un-stimulated (notice CCR2B receptors at the plasma membrane) or stimulated with 20 nM CCL2 for 30 min were fixed and permeabilized before incubation with M1 anti-Flag and secondary anti-mouse Alexa 568. Images were mounted on DAPI-ProlongGold (Life Technologies). Imaging was performed with a LeicaTCS SP5 using a 63x 1.3 NA oil immersion lens. (C) Confocal micrographs of shFLNa28 and shScr CCR2B expressing HeLa cells incubated with 20 nM CCL2 for 15 min at 37°C, fixed and immunostained with anti-EEA-1 antibodies followed by Alexa 488 and M1 anti-Flag antibodies, then Alexa 568, washed and mounted with Prolong. A representative image of one z-layer is shown. Scale bars 10 μm.
Fig. S2 (A) The fluorescence recovery of Rab4A in endosomes is not affected in FLNa defective HEK293 cells. Time series of fluorescence micrographs showing FRAP of Rab4A-YFP-loaded endosomes in controls (shscr) and shFLNa HEK293 cells transduced with shFLNa or shScr and transiently transfected with Rab4A-YFP. Cells were incubated at 37°C in a CO₂ chamber. The 514 nm line was used for bleaching an area enclosing the entire endosome (4 μm) and monitoring of Rab4A-YFP-fluorescence recovery during for 10 s. Images were recorded as 16-bit sequences with a pinhole of 246 μm, giving a z slice of 2 μm. Images were acquired with a Leica TCS SP5 using a 63x 1.3 NA oil immersion lens at 3 s intervals. (B) HEK293 transiently transfected with Rab4A-YFP and Flag-V2R, Flag-ETB or Flag-PAR1 were pre-labeled with anti-Flag and Alexa 647 anti-mouse, induce to internalize with addition of the corresponding ligands and in vivo imaged. Zooming on individual endosomes. Images were acquired as in Figure 3A and quantified in Figure 3B.
Fig. S3. Expression pattern of FLNa, FLNa-S2152A and FLNa-S2152E. (A) Representative confocal images (life images) of endosomal structures of HeLa cells transiently transfected with Rab4A-YFP in the presence of mFLNa-wt-DsRed, mFLNa-S2152E-DsRed and mFLNaABD-DsRed. Imaging was taken with a Leica TCS SP5 using a 63x 1.3 NA oil immersion lens. Scale bars 5 μm. (B) FLNa-S2152E-GFP and FLNa-S2152A-GFP are present in cortical actin structures. Confocal micrographs of HeLa cells transiently transfected with FLNa-S2152E-GFP or FLNa-S2152A-GFP. A day after transfection cells were fixed and imaged with a Leica TCS SP5 using a 63x 1.3 NA oil immersion lens. Images show two different z-planes corresponding to upper cell layers (cortex) (left images) or medium cell layers (showing cell membrane) (right images). Scale bars 10 μm. (C) FLNa-S2152E-GFP and FLNa-S2152A-GFP HEK293 are present in cortical actin structures. HEK293 cells transiently transfected with FLNa-S2152E-GFP or FLNa-S2152A-GFP and stained with Phalloidin555 (Life Technologies) for 30 min at room temperature at a 1:250 dilution. Scale bars 10 μm. Arrows indicate cortical structures, arrow heads, microdomains in vesicular structures.
Fig. S4. CCL2 stimulation of pERK. (A) Immunoblot against phospho-ERK (pERK) or ERK from protein extract from shFLNa or scr HeLa cells expressing Flag-CCR2B. Cells were stimulated with 20 nM CCL2 for different times. Lysates were immunoblotted with anti-phosphorylated ERK (top) and reprobed with anti-ERK (bottom). shFLNa +FLNa indicated cells depleted of human FLNa and expressing mouse shFLNa-insensitive FLNa. Data (quantified in the linear range with Odyssey) normalized for total ERK levels are expressed as the average fold-increase ± SEM of four independent experiments. (B) Shows two different immunoblot analysis of ERK activation by CCL2 in shRNA and scr HeLa cells trasiently transfected with CCR2B. Analysis was done as in A.
Supplementary Movies

**Movie S1** Time series showing FRAP of CCR2B-loaded ensosomes in control src- and shFLNa cells. HEK293 cells transduced with src- and shFLNa expressing Flag-CCR2B (with the LUT rainbow of FIJI) and Rab4-YFP surface labeled were treated as in Figure 2. A whole endosome was bleached at a time and imaged during 2.5 min.
**Movie S2** Time series showing FRAP of Rab4 ensodomes in control src- and shFLNa cells. HEK293 cells transduced with scrRNA and shFLNa expressing Rab4-YFP. A whole endosome was bleached at a time and imaged during 15 s.
**Movie S3** Time-lapse acquisition of HeLa cells expressing Flag-CCR2B (white) and FLNa-GFP (green) and Rab4-Cherry (red) acquired as in Figure 3. HeLa cells were transfected with the corresponding plasmids, pre-labeled with anti-Flag followed by Alexa647 at anti-mouse antibody 4°C and immediately followed by live confocal imaging after 20 nM CCL2. Representative vesicles are shown from a single cell.
**Movie S4** Time-lapse acquisition of HeLa cells expressing Flag-CCR2B (white) and FLNa-GFP (green) and Rab5-Cherry (red) acquired as in Figure 3. HeLa cells were transfected with the corresponding plasmids, pre-labeled with anti-Flag followed by Alexa647 at anti-mouse antibody 4°C and immediately followed by live confocal imaging after 20 nM CCL2. Representative vesicles are shown from a single cell.
**Movie S5** Time-lapse acquisition of HeLa cells expressing Flag-CCR2B (white) and FLNa-GFP (green) and Rab11-Cherry (red) acquired as in Figure 3. HeLa cells were transfected with the corresponding plasmids, pre-labeled with anti-Flag followed by Alexa647 at anti-mouse antibody 4°C and immediately followed by live confocal imaging after 20 nM CCL2. Representative vesicles are shown from a single cell.