Etude de l’interaction entre EFA6 et l’endophiline

I. Contexte et objectif de l’étude

L’équipe « les protéines Arf, morphologie cellulaire et transport membranaire », dans laquelle j’ai effectué mon doctorat, étudie depuis de nombreuses années le rôle du facteur d’échange EFA6 et de son substrat Arf6 dans différents processus cellulaires. Ainsi ces deux protéines ont pu être impliquées dans la réorganisation du cytosquelette d’actine, dans la régulation du transport vésiculaire et dans la mise en place de la polarité. Dans le but d’approfondir nos connaissances sur les fonctions d’EFA6, une recherche de partenaires a été effectuée par une expérience de double hybride chez la levure utilisant le domaine Sec7 d’EFA6 comme appât. Par cette méthode plusieurs clones codant pour l’endophiline B1 ont pu être identifiés.

Dans un premier temps mon travail a porté sur la validation de cette interaction entre EFA6 et l’endophiline dans des cellules et in vitro, et sur l’identification des domaines impliqués. Par la suite je me suis intéressée au rôle de cette interaction sur la fonction de chacune des deux protéines : l’activité catalytique d’EFA6 sur l’échange nucléotidique d’Arf6 et la capacité de l’endophiline à lier les membranes et induire la formation de tubules. Pour finir j’ai étudié la localisation cellulaire de ces deux partenaires et le rôle fonctionnel de cette interaction dans l’internalisation du récepteur à la transferrine.

Dans le but de valider cette interaction nous avons utilisé une approche biochimique avec des expériences de pull-down sur des lysats cellulaires sur-exprimant nos protéines d’intérêt ainsi que sur des protéines recombinantes purifiées. Nous avons également réalisé des expériences de flottaison sur gradient de sucrose à l’aide de vésicules lipidiques artificielles avec des degrés de courbure compatibles avec une membrane plane ou avec un puits d’endocytose en formation. Lors des expériences de flottaison, les protéines d'intérêt sont incubées en présence de liposomes puis un gradient de sucrose est déposé sur ce mélange. Suite à une ultracentrifugation, les liposomes de faible densité se retrouvent dans la fraction

«top» correspondant à la partie supérieure du gradient de sucrose. Les protéines capables d'interagir avec les liposomes sont elles aussi entrainées dans la fraction «top», les autres quant à elles restent dans la fraction «bottom». A cela ce sont ajoutées des expériences de biologie cellulaire et de microscopie électronique.

L'ensemble de cette étude nous a permis de proposer un modèle rendant compte du rôle de l'interaction entre l'endophiline et EFA6 dans l'endocytose dépendante de la clathrine.

II. Article

Arf6 exchange factor EFA6 and endophilin directly interact at the plasma membrane to control clathrin-mediated endocytosis

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Members of the Arf family of small G proteins are involved in membrane traffic and organelle structure. They control the recruitment of coat proteins, and modulate the structure of actin filaments and the lipid composition of membranes. The ADP-ribosylation factor 6 (Arf6) isoform and the exchange factor for Arf6 (EFA6) are known to regulate the endocytic pathway of many different receptors. To determine the molecular mechanism of the EFA6/Arf6 function in vesicular transport, we searched for new EFA6 partners. In a two-hybrid screening using the catalytic Sec7 domain as a bait, we identified endophilin as a new partner of EFA6. Endophilin contains a Bin/Amphiphysin/Rvs (BAR) domain responsible for membrane bending, and an SH3 domain responsible for the recruitment of dynamin and synaptojanin, two proteins involved, respectively, in the fission and uncoating of clathrin-coated vesicles. By using purified proteins, we confirmed the direct interaction, and identified the N-BAR domain as the binding motif to EFA6A. We showed that endophilin stimulates the catalytic activity of EFA6A on Arf6. In addition, we observed that the Sec7 domain competes with flat but not with highly curved lipid membranes to bind the N-BAR. In cells, expression of EFA6A recruits endophilin to EFA6A-positive plasma membrane ruffles, whereas expression of endophilin rescues the EFA6A-mediated inhibition of transferrin internalization. Overall, our results support a model whereby EFA6 recruits endophilin on flat areas of the plasma membrane to control Arf6 activation and clathrin-mediated endocytosis.

small GTP-binding proteins | membrane curvature | vesicular trafficking

The ADP ribosylation factor family, which includes six members, is known to regulate different stages of vesicular trafficking (reviewed in refs. 1, 2). The most abundant isoform, Arf1, controls the membrane trafficking at the level of the Golgi apparatus by regulating in a GTP-dependent manner the recruitment of the COPI coat complex (3–5) and the two clathrin adaptors AP-1 (6, 7) and GGAs (8) onto the Golgi membranes. By activating lipid-modifying enzymes, Arf1 is able to change the lipid composition of the donor compartment membrane, thus facilitating membrane dynamic (9–12). ADP-ribosylation factor 6 (Arf6), the most distant isoform, is thought to regulate plasma membrane and endosomal trafficking. Similarly to Arf1, Arf6 activates the PLD (13, 14) and the type I PI4P5K (15, 16) to produce, respectively, phosphatidic acid, which is a fusogenic lipid, and phosphatidylinositol 4–5 bisphosphate, which is known to regulate clathrin-dependent endocytosis. Arf6 and PIP_2 cooperate (at least in vitro) to recruit AP-2 onto lipid membranes, suggesting a role for Arf6 in the formation of clathrin-coated pits (17). In addition to this putative role during the initial steps of internalization, Arf6 has been shown to interact and recruit Nm23-H1, a protein believed to control the dynamin-dependent fission of endocytic vesicles (18). These different observations have clarified the molecular basis of the role of Arf6 in clathrin-dependent and -independent endocytosis. Arf6 has been shown to be involved in the internalization of different cargos, such as β1 integrin, E-cadherin, MHC class I, G protein-coupled receptors, and poly-Ig receptor (19–22). Moreover, EFA6, the Arf6 specific exchange factor, has been shown recently to activate Arf6 in response to β2AR stimulation in a β-arrestin–dependent manner (23). This ligand-mediated activation of Arf6 couples stimulation and Arf6-dependent trafficking of the G protein-coupled receptor. In addition to its role in internalization, Arf6 also seems to be required for the recycling of endosomes. Indeed, activated Arf6 controls the fast recycling of the transferrin receptor (Tfn-R) via two effectors, JIP3/4 (24) and the Sec10 subunit of the exocyst (25), and the recycling of the β2AR, probably through Rab4 activation (23). Also, Arf6 has been involved in the recycling of the IL2 receptor α-subunit, syndecan, integrin β1, and MHC class I (19, 26–28).

EFA6 belongs to the Sec7 domain-containing protein family that acts as guanine nucleotide exchange factor (GEF) for Arf proteins (reviewed in ref. 29). In humans, the EFA6 protein family contains four isoforms, and shares a common domain organization consisting of a Sec7 domain bearing the catalytic activity, a pleckstrin homology domain responsible for the plasma membrane localization by interacting with PIP_2 and F-actin, a C-terminal region containing a putative coiled-coil motif and two proline rich motifs responsible for F-actin reorganization, and an N-terminal domain of unknown function whose size and primary sequence are the least conserved across the four isoforms. EFA6 is highly selective for Arf6, and is known to coordinate plasma membrane trafficking with actin cytoskeleton remodeling (30). EFA6 interacts directly

Significance

The small G protein ADP-ribosylation factor 6 (Arf6) and the exchange factor for Arf6 (EFA6) are involved in endocytic vesicular transport, but their precise functions remain unclear. The Bin/Amphiphysin/Rvs (BAR) domain containing endophilin is known to couple fission to uncoating of the clathrin-coated vesicles. Here, we identified endophilin as a direct interactor of EFA6. We analyzed in vitro the effect of this interaction on EFA6 guanine nucleotide exchange factor activity, and on endophilin lipid binding and remodeling activities. We then studied in vivo the role of the two proteins in transferrin receptor endocytosis. Our results suggest a model in which EFA6 recruits endophilin on flat areas of endocytic zones of the plasma membrane, where endophilin cooperates with EFA6 to activate Arf6 and regulate clathrin-mediated endocytosis.

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with F-actin (31) and α-actinin (32), and its overexpression leads to the formation of F-actin rich microvilli at the plasma membrane (30, 33). EFA6 is involved in the endocytic/recycling transport of several membrane proteins such as the Tfn-R, β2AR and K^+ channel Twik1, and in the assembly of the tight junction in epithelial cells (23, 34–36). However, even though EFA6 functions are starting to be uncovered, little is known regarding its regulation.

Endophilins are members of the large and heterogeneous family of Bin/Amphiphysin/Rvs (BAR) domain proteins. They are encoded by endophilin 1–3 (i.e., A1-3) and B1-2 genes (reviewed in ref. 37). They function in synaptic vesicle recycling, membrane receptor trafficking, and different processes that require membrane remodeling. Endophilin BAR domain is part of the N-BAR subfamily (that contains an N-terminal amphipathic helix) that folds into a crescent-shaped dimer able to sense membrane curvature in vitro and to induce the deformation and tubulation of liposomes (reviewed in refs. 38, 39). The N-terminal BAR domain of endophilin is followed after a short linker by a C-terminal SH3 domain. This SH3 domain has been shown to bind dynamin and synaptojanin, two proteins involved in endocytic vesicle scission and uncoating, respectively (40–42). Although endophilin has been extensively investigated in vitro, its precise role in the cell has yet to be clarified.

To unravel at the molecular level the function of the EFA6/Arf6 pathway in membrane trafficking, we have looked for new partners of EFA6. Here, we identified the endophilin N-BAR domain as a direct interactor of the EFA6A Sec7 domain. We analyzed in vitro the effect of this interaction on the GEF activity of EFA6A, and on the lipid binding and remodeling activities of endophilin. We then studied in vivo the role of the two proteins in the endocytosis of the Tfn-R. Our results suggest a model in which EFA6 recruits endophilin on flat areas of endocytic zones of the plasma membrane, where endophilin cooperates with EFA6 to activate Arf6 and to regulate clathrin-mediated endocytosis.

Results

Endophilin Interacts with EFA6A. To uncover regulators of the nucleotide exchange factor activity of EFA6A, we performed a yeast two-hybrid screen by using its catalytic Sec7 domain as a bait. We identified several clones encoding for endophilin B1. GST pull-down experiments using lysates from transfected cell confirmed the interaction and showed that EFA6A could interact with endophilin A1, A2, and B1 isoforms (Fig. S1). From these experiments, we concluded that EFA6A interacts with members of the two endophilin subfamilies A and B.

Endophilin Directly Interacts with EFA6A and Stimulates Its Nucleotide Exchange Activity for Arf6. Next, we used purified recombinant proteins and different constructs of endophilin A1 (Fig. 1*A*) to show that His-EFA6A interacts directly with the first 125 aa of the N-BAR domain (1/2-N-BAR; Fig. 1*B*), known to retain the activity of lipid binding and tubule formation (43). As the Sec7 domain of EFA6A was used as bait in the two-hybrid screen, we confirmed that the purified recombinant Sec7 domain interacted with the GST-N-BAR (Fig. 1*C*). Moreover, we observed that the deletion of the N-terminal helix (residues 1–36) of the N-BAR did not abolish the interaction with the Sec7 domain (Fig. 1*D*). Considered together, these data demonstrated a direct and specific binding of the Sec7 domain of EFA6A to a subdomain (residues 36–125) of the N-BAR domain of endophilin. We then studied the specificity of the interaction by using two other purified BAR domain-containing proteins. In contrast to GST-endophilin, neither GST-arfaptin nor GST-amphiphysin were able to interact directly with His-EFA6ASec7 (Fig. S2). It suggests that the primary sequence, together with the 3D structure, is responsible for the interaction with EFA6A-Sec7 domain.

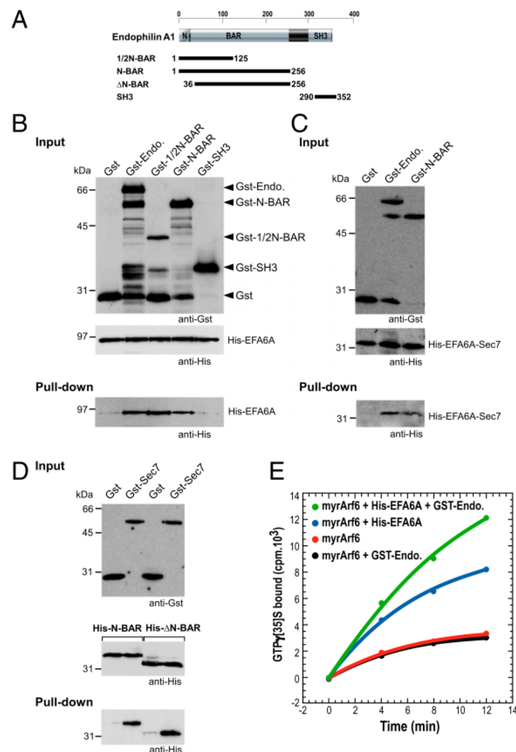

Fig. 1. Endophilin directly interacts with EFA6 and stimulates its GEF activity on Arf6. (*A*) Schematic representation of endophilin A1 and the different GST constructs used in this study. (*B* and *C*) GST pull-down of purified His-tagged EFA6A (*B*) or EFA6A-Sec7 domain (*C*) by different constructs of endophilin A1 fused to GST. (*D*) GST pull-down of purified His-tagged N-BAR or ΔN-BAR domain by EFA6-Sec7 domain fused to GST. (*E*) Kinetics of [^{35}S] GTPγS binding to purified and myristoylated Arf6 (2 μM) were measured (*Methods*) in the presence of phospholipid vesicles and in the presence or the absence of purified His-tagged EFA6A (~200 nM) and endophilin A1 fused to GST (2 μM). The results are representative of at least three independent experiments.

As endophilin interacted with the catalytic Sec7 domain, we investigated whether it could modulate the GEF activity of EFA6A on Arf6. We examined in vitro the kinetics of the spontaneous and His-EFA6A catalyzed Arf6 activation in the presence or absence of GST-endophilin (Fig. 1*E*). We carried out our assay in conditions closest to normal physiology; that is, at the surface of large liposomes and by using myristoylated Arf6. The presence of endophilin did not affect the spontaneous activation of Arf6, whereas it strongly stimulated that catalyzed by EFA6A. These results suggested first that EFA6A binds simultaneously Arf6 and endophilin, and second that, by interacting directly with the Sec7 domain, the endophilin acts as a positive regulator of the GEF activity.

The EFA6A-Sec7 Domain Competes with Lipids to Bind the N-BAR Domain. As N-BAR domains are essentially known to interact with curved lipid membranes, we examined whether the Sec7 domain could affect the binding of endophilin N-BAR domain to lipid vesicles of different sizes. To evaluate in a direct manner

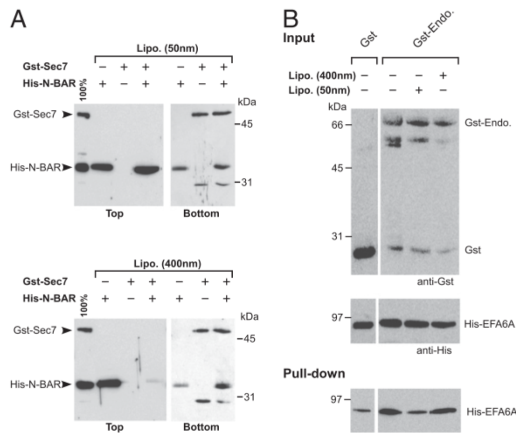

Fig. 2. EFA6-Sec7 domain and highly curved lipid membranes compete to bind endophilin N-BAR domain. (*A*) Flotation assay. EFA6A-Sec7 fused to GST (1.5 μM) or His-tagged endophilin N-BAR (4 μM) or a mixture of the two proteins were incubated with small (50 nm) or large (400 nm) phospholipid liposomes (1 mM). The sample suspension was adjusted to 30% sucrose and then overlaid with two cushions of decreasing sucrose density (*Methods*). After centrifugation, the top (lipids and associated proteins) and bottom (unbound material) fractions were analyzed by SDS/PAGE. Proteins were stained with SYPRO Orange. (*B*) GST pull-down of purified His-tagged EFA6A by endophilin A1 fused to GST in the absence or the presence of small (50 nm) or large (400 nm) liposomes. The results shown are representative of at least three independent experiments.

the binding of endophilin N-BAR and EFA6A-Sec7 to liposomes, we used a flotation assay in which the lipids and the associated proteins are recovered by centrifugation at the top of a sucrose gradient. We tested two sizes of liposomes: large liposomes with a diameter of ∼0.4 μm mimicking flat membranes, and small liposomes (∼0.05 μm in diameter) simulating endocytic vesicles. Although the affinity of the N-BAR is known to be higher for curved membranes, in our conditions, we found the N-BAR to be associated with both kinds of vesicles, whereas, as expected, the Sec7 domain did not bind to either liposomes (Fig. 2*A*). Surprisingly, in the presence of the Sec7 domain, the N-BAR was found to be associated only with small liposomes, indicating that the Sec7 domain inhibited the binding of the N-BAR to the large liposomes. These data demonstrated that the endophilin N-BAR could not interact simultaneously with lipid membranes and EFA6A-Sec7. These results were strengthened by a GST pull-down experiment using purified recombinant proteins in the presence of liposomes. Fig. 2*B* shows that GST-endophilin recovered EFA6A even in the presence of large liposomes (∼0.4 μm). However, in the presence of small vesicles (∼0.05 μm), GST-endophilin was not able to retain EFA6A (Fig. 2*B*).

Overall, these data demonstrated that the EFA6A-Sec7 domain competes with lipids for binding to the N-BAR. Furthermore, they demonstrated that, under our experimental conditions, highly curved lipid membranes dissociate the EFA6A-Sec7/N-BAR complex, whereas the Sec7 domain disrupts the interaction of N-BAR with flat membranes. In other words, the interaction between EFA6 and endophilin is regulated by the membrane curvature.

The Endophilin N-BAR–Induced Deformation and Tubulation of Large Liposomes Is Inhibited by the EFA6A-Sec7 Domain. We then asked whether EFA6A-Sec7 could affect the function of the endophilin N-BAR by looking at its ability to induce the deformation and tubulation of liposomes. By dynamic light scattering, it is possible to determine the radius [i.e., hydrodynamic radius (Rh)] of the liposomes in solution. Fig. 3*A* shows that the presence of the N-BAR (Fig. 3*A*, blue diamonds) induced an increase over time of the Rh of large (Fig. 3*A*, *Left*) and small (Fig. 3*A*, *Right*) lipid vesicles in comparison with vesicles alone (Fig. 3*A*, yellow triangles), verifying that the N-BAR affects the form and the size of the liposomes. We used negative stain EM to visualize the change of size and to determine the nature of the deformation. As shown in Fig. 3*B*, a 20-min incubation of purified N-BAR domain with large liposomes led to a strong tubulation of nearly all liposomes, which then disappeared or became much smaller (Fig. 3*B*, *Center*). Interestingly, we observed that the addition of the Sec7 domain (Fig. 3*B*, pink squares) inhibited the N-BAR–dependent increase of the Rh of only the large liposomes (Fig. 3*A*). This result was confirmed by EM analysis. When the Sec7 and the N-BAR domains were added to the large lipid vesicles, only few tubules were formed, and numerous liposomes looked similar to the control ones in terms of size and shape (Fig. 3*B*, *Right*).

Thus, collectively, these results indicated that EFA6A-Sec7, by interacting with the endophilin N-BAR, inhibits its ability to generate membrane curvature. However, when membrane curvature is high, the Sec7 does not interfere with N-BAR–dependent tubulation. These observations confirmed that the Sec7 domain could not displace the complex of the N-BAR domain with highly curved membrane.

EFA6A Induces the Recruitment of Endophilin to the Plasma Membrane. We then investigated the biological function of the EFA6/endophilin interaction. First, we examined the localization of both proteins in HeLa cells by immunofluorescence. As previously published (30, 31), EGFP-EFA6A localized essentially to the plasma membrane, and particularly in membrane ruffles and F-actin–rich microvilli-like structures induced upon its expression (Fig. 4*A*). In contrast, myc-tagged endophilin A2 was found

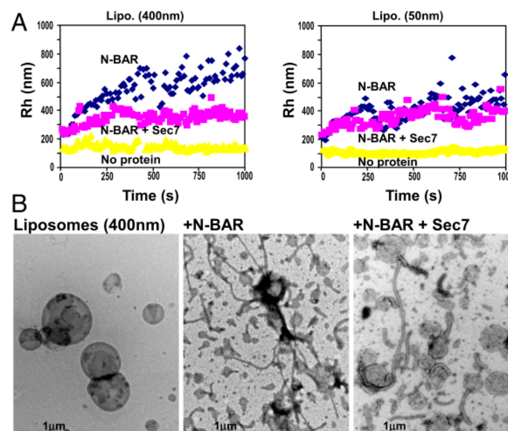

Fig. 3. EFA6-Sec7 inhibits the endophilin N-BAR–induced aggregation and tubulation of large liposomes. (*A*) Effect of His N-BAR and GST-EFA6A-Sec7 on the aggregation and tubulation of liposomes. Large (400 nM, *Left*) or small (50 nM, *Right*) liposomes incubated alone (yellow triangles) or in the presence of His-N-BAR (blue diamonds) or His-N-BAR and GST-EFA6A-Sec7 (pink squares) were analyzed in real time by dynamic light scattering (*Methods*). (*B*) EM image of large liposomes (400 nm) incubated alone (*Left*) or in the presence of His-N-BAR (*Center*) or His-N-BAR and GST-EFA6A-Sec7 (*Right*). (Scale bar, 1 μM.) The results shown are representative of three separate experiments.

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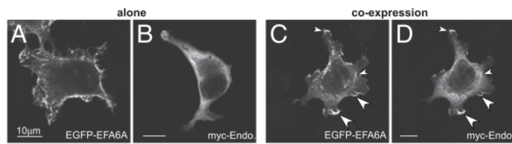

Fig. 4. Coexpression with EFA6 induces the redistribution of endophilin to the plasma membrane. BHK-21 cells were transfected with EGFP-EFA6A (*A*) or with myc-endophilinA2 (*B*) or both (*C* and *D*). After fixation, the cells were processed for immunofluorescence (*Methods*). The images shown are representative of at least five separate experiments.

mostly in the cytoplasm (Fig. 4*B*). When the proteins were coexpressed (Fig. 4 *C* and *D*), we noticed that a large portion of endophilin was found in EFA6A-positive structures of the plasma membrane, suggesting that EFA6A recruits endophilin to the plasma membrane. It should be noted that the EFA6A-induced structures were not affected by the presence of endophilin. Thus, the direct interaction described in vitro between the Sec7 domain of EFA6A and the N-BAR domain of endophilin could account for the EFA6A-mediated recruitment of endophilin to the plasma membrane. Furthermore, in light of our in vitro results, the interaction should preferentially occur in flat zones of the plasma membrane. Regardless, these data identified endophilin as a novel EFA6-interacting protein at the plasma membrane.

Endophilin Rescues the EFA6A-Induced Inhibition of Transferrin Internalization. We have previously shown that the overexpression of EFA6A inhibited Tfn uptake and caused redistribution of the Tfn-R to the cell surface by an unknown mechanism (30). In light of our new results described here, and because endophilin is known to be a key regulator of clathrin-dependent endocytosis, one explanation could be that the overexpressed EFA6A inhibited the formation of endocytic vesicles, and thereby Tfn internalization, by sequestering the endogenous endophilin on flat regions of the plasma membrane. To test this hypothesis, we examined the internalization of fluoresceinated human Tfn in TRV-b1 cells [a CHO-derived cell overexpressing the human Tfn-R (44)] expressing EFA6A, endophilin A2, or both (Fig. 5). In untransfected cells, Tfn internalized for 3 min was found throughout the cells in small vesicular structures known to be early endosomes (Fig. 5*A*, *Left*), whereas, after 30 min, it was mostly accumulated in a single large patch in the pericentriolar area known to be the endocytic recycling compartment (Fig. 5*A*, *Right*). As previously published, we observed that overexpression of endophilin A2 had no effect on the rate of Tfn internalization. In contrast, overexpression of EFA6A strongly inhibited Tfn uptake as strongly at 3 min as at 30 min, with a ~50% inhibition being measured (Fig. 5). However, when cells were cotransfected with EFA6A and endophilin, they internalized Tfn similarly to untransfected or endophilin-expressing cells (Fig. 5). Thus, overexpression of endophilin rescued the inhibition of the Tfn uptake mediated by EFA6A.

Altogether, these results showed that EFA6A and endophilin are part of the same pathway that controls Tfn-R internalization.

Discussion

In this study, we identified and characterized a direct interaction of EFA6, the Arf6-specific exchange factor, with endophilin, a protein involved in clathrin-coated vesicle formation. This interaction, which is, to our knowledge, the first described between an ArfGEF and an N-BAR–containing protein, clearly reinforces the link between the EFA6/Arf6 pathway and endocytic vesicular transport.

Until now, the factors that trigger endophilin recruitment to the membrane have been unknown. Here, we observed that endophilin A2 and B1, representative members of the two endophilin subfamilies that are normally found diffused throughout the cytosol, are strongly recruited to the specific EFA6-enriched domains of the plasma membrane when coexpressed with EFA6. This observation indicates that EFA6 contributes to the plasma membrane localization of endophilin. Thus, our results identified and characterized EFA6 as one of the factors that recruit endophilin to the plasma membrane.

Nevertheless, the EFA6/endophilin interaction is probably not constitutive. Besides the membrane curvature that induces the dissociation of the Sec7/N-BAR complex, other regulatory signals must exist to control this interaction. A better understanding of the EFA6/endophilin pathway will require the identification of the signals and the molecular mechanisms that regulate in time and space the formation of the complex. It has been proposed that phosphorylation of a couple of sites in the N-BAR domain could modulate the endocytic function and membrane binding properties of endophilin (ref. 45; reviewed in ref. 37). It will be of interest to study if endophilin phosphorylation can also modulate the interaction of the N-BAR domain with EFA6.

Numerous proteins involved in intracellular vesicular transport have been shown to interact with phospholipid membranes in a membrane curvature-dependent manner. This dependency is given by sensor domains such as amphipathic helix, Alps motif, BAR domain. However, to our knowledge, we have now characterized for the first time a protein–protein interaction that is

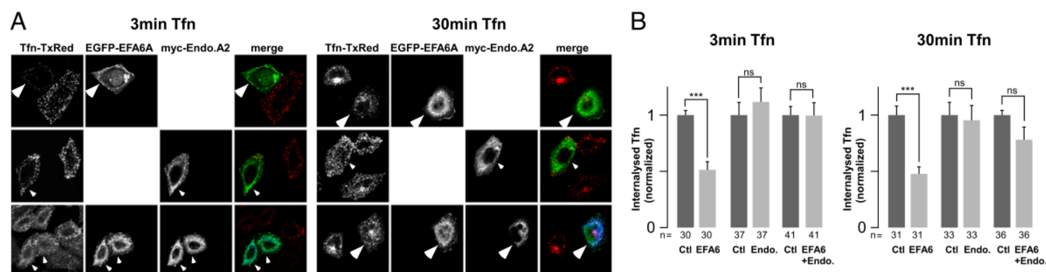

Fig. 5. Expression of endophilin rescues the EFA6-induced inhibition of transferrin uptake. (*A* and *B*) TRVb-1 cells expressing EGFP-EFA6, myc-endophilin A2, or both were incubated at 37 °C for 3 min (*Left*) or 30 min (*Right*) with iron-saturated Texas red-conjugated human transferrin, fixed, and processed for immunofluorescence (*A*). Arrowheads indicate the transfected cells. Internalized transferrin was quantified (*B*) as described in *Methods* and normalized to the amount of internalized transferrin in untransfected cells in each condition (error bars represent SEM, *n* indicates the number of analyzed cells). Statistical analysis was performed by Student *t* test. ns, not significant, i.e., $P > 0.1$ (***$P < 0.001$).

controlled by membrane curvature. According to our in vitro results, the recruitment of endophilin by EFA6 will occur only on flat areas of the plasma membrane. Indeed, the interaction between the catalytic Sec7 domain of EFA6 and the lipid-binding N-BAR domain of endophilin was down-regulated by the membrane curvature. Also, it implies that the Sec7 domain binding site is located in the concave face of the endophilin N-BAR that is known to interact with the lipid membranes (46).

What could be the function of this EFA6-induced plasma membrane recruited endophilin? Here we demonstrated that endophilin binds to the catalytic Sec7 domain to simulate Arf6 activation. Endophilin constitutes the first activator of the catalytic activity of EFA6 by directly interacting with its Sec7 domain. We have previously reported that in response to isoproterenol, a β2 adrenergic receptor agonist, β-arrestin was able to stimulate the EFA6-mediated activation of Arf6. However, this stimulation occurs probably by a different mechanism from that used by endophilin. Indeed, β-arrestin interacts simultaneously with EFA6 and Arf6GDP to corecruit the GEF and its substrate. Thus, β-arrestin stimulates Arf6 activation by reducing the dimensionality. An interaction with the N-BAR domain of arfaptin has been reported with Arf and Arl proteins (47, 48). However, we did not observe a direct interaction of Arf6 with endophilin. Thus, we hypothesize that endophilin modulates the GEF activity of EFA6 by affecting the 3D structure of the Sec7 domain, or by affecting the overall structural organization of the protein, leading to an increase in its affinity for Arf6. Structural analysis would be required to identify precisely the binding site and the mechanism by which endophilin enhances the GEF activity.

Regardless of the mechanism, the endophilin recruited to the plasma membrane by EFA6 would increase the pool of activated Arf6GTP. However, this stimulatory effect would occur only in the absence of a highly curved membrane. Indeed, we observed dissociation of the Sec7/N-BAR complex in the presence of small lipid vesicles, suggesting a chronological sequence of events. First, EFA6 would recruit endophilin to a flat zone of the plasma membrane, where both proteins would cooperate to activate Arf6. A high concentration of Arf6GTP will then activate the type I PIP5Kinase, recruit AP-2 and the clathrin molecules to shape the membrane, and build the clathrin pits. The increasing membrane curvature of the clathrin-coated vesicle will then produce the dissociation of the Sec7/N-BAR complex, releasing the endophilin, which can then assume its functions in endocytic vesicle fission and uncoating.

In addition, it is intriguing that EFA6 was found to interact directly or indirectly (via adaptors) with cargos and regulate their intracellular distribution. We previously demonstrated that EFA6 binds to the stimulated β2AR through β-arrestin to control its intracellular fate (23). We have also established that EFA6 interacts directly with TWIK-1, a K^+ channel, to govern its distribution between the plasma membrane and the recycling endosomes (36). From these observations, we propose that EFA6 acts as a platform to connect the cargo to the endocytic molecular machinery, notably by controlling directly different steps from coat assembly to vesicle scission.

We observed that EFA6 can interact with the three different isoforms that we have tested, namely endophilin A1, A2, and B1. Endophilins A1 and A2 are known to act in the final stages of endocytosis by recruiting dynamin and synaptojanin for clathrin vesicle fission and uncoating, respectively. This suggests that EFA6 and Arf6 may be involved in the control of clathrin-dependent endocytosis. A role that is in agreement with their principal localization at the plasma membrane, with the effect of their overexpression on transferrin internalization, and with the capability of Arf6 to stimulate PIP2 formation and AP-2 recruitment. Endophilin B1 (which shares approximately 30% identity with A1) also exhibits lipid binding and liposome tubulation properties. However, in contrast to the A isoforms, B1 is found associated with intracellular organelles, and particularly with early endosomes, in which it colocalizes and forms a complex with EEA1. In addition, B1 has been involved in the recycling of the neurotrophin nerve growth factor (i.e., NGF/TrkA; reviewed in ref. 49). Although we have never found EFA6 localized to early endosomes, there is much evidence for involvement of Arf6 in receptor recycling. Arf6 has been involved in the recycling of different receptors (e.g., Tfn receptor, β2AR, IL-2 receptor) and intracellular EEA1-positive staining has also been reported for activated Arf6 (23, 27). One cannot exclude that EFA6 participates in endophilin B1 function at the early endosome level, and further studies are required to analyze such a role in receptor recycling.

In summary, our results indicate that EFA6 and endophilin act as key regulators of receptor-mediated endocytosis, and cooperate to regulate clathrin-dependent endocytosis.

Methods

DNA Constructs. Sequences encoding residues 1–125 (1/2-N-BAR), 1–256 (N-BAR), and 290–352 (SH3) of mouse endophilin A1 were obtained by PCR and cloned into pGEX-3X (GE Healthcare) for in-frame fusion with GST at the N terminus. Sequences encoding residues 1–256 (N-BAR) and 36–256 (ΔN-BAR) of mouse endophilin A1 were obtained by PCR and cloned into pET16b (Novagen) for in-frame fusion with hexa-His tag at the N terminus. Plasmids encoding vsv-g–tagged EFA6A, EGFP-EFA6A, His-EFA6A, His-EFA6A-Sec7, GST-EFA6A, GST-EFA6A-Sec7, and His-Arf6 have been described elsewhere (30, 33, 50). pcDNA3 myc-tagged mouse endophilin A2, pcDNA3 myc-tagged human endophilin B1, and pGEX-6p-mouse endophilin A1 were provided by A. Schmidt (Institut Jacques Monod, Paris, France). pGEX-human arfaptin-2 and pGEX-human amphiphysin II were provided by P. De Camilli (Yale University, New Haven, CT).

Expression and Purification of Recombinant Proteins. For the in vitro binding assays, recombinant myristoylated Arf6 WT with a C-terminal hexa-his tag (MyrArf6) was prepared as described elsewhere (25, 51, 52). Recombinant his-tagged EFA6A (His-EFA6A) was prepared as previously described (31). The different GST fusion proteins were produced in *Escherichia coli* and purified by affinity chromatography on glutathione–Sepharose beads (GE Healthcare). After elution with glutathione, the purified proteins were dialyzed against 20 mM Tris·HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 10% glycerol (dialysis buffer), and stored at −20 °C. Recombinant His-tagged N-BAR and ΔN-BAR domains were expressed in *E. coli* and purified according to the manufacturer's instructions (Qiagen).

Internalization of Texas Red-Conjugated Transferrin. TRVb-1 cells plated on 11-mm round glass coverslips were transiently transfected with plasmids encoding EGFP-tagged EFA6A and myc-tagged endophilin A2 as indicated, by using the Jet Pei transfection reagent as described by the manufacturer. Twenty-four hours after transfection, cells were preincubated in serum-free medium containing 1% BSA for 30 min at 37 °C, and then incubated for 3 or 30 min in the same medium supplemented with 50 μg/mL of Texas red-conjugated human transferrin (Molecular Probes/Fisher Scientific). Cells were then washed twice in ice-cold PBS solution, fixed in 3% (wt/vol) paraformaldehyde, and processed for immunofluorescence analysis as described previously (53). The confocal images obtained from the laser scanning confocal microscope (TCS SP5; Leica Microsystems) were analyzed to quantify the transferrin uptake by using a homemade ImageJ (http://imagej.nih.gov/ij/) macro program (W. S. Rasband). Briefly, each cell contour was determined on images corresponding to EGFP-EFA6A–expressing cells, and all the others on Texas red transferrin images by segmentation (filtering and thresholding followed by a watershed on the binary image and detection of the objects). The cumulated surface and intensities of the intracellular transferrin granules and their number were determined in both populations on each cell by using the region of interest defined by the contour.

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Supporting Information

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SI Methods

Cell Culture, Reagents, and Antibodies. HeLa cells were grown in HeLa media corresponding to DMEM (Invitrogen) containing 10% FCS (HyClone/Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen). TRVb-1 cells were grown in Ham F-12 medium (Invitrogen), 5% FCS, penicillin/streptomycin, and 100 μg/mL G418 (Invitrogen). The following were obtained from Jackson ImmunoResearch: mouse mAb against vsv-g epitope (clone P5D4; Roche Diagnostics), mouse mAb against His epitope (Sigma-Aldrich), mouse mAb against myc epitope (clone 9E10; Roche Diagnostics), FITC and Texas red conjugated antibodies. Iron-loaded Texas red-conjugated human transferrin was from Molecular Probes/Fisher Scientific. Asolectin, unlabeled nucleotides, and egg dioleyl-phosphatidylcholine were from Sigma. Liver phosphatidylethanolamine and brain phosphatidylserine were from Avanti Polar Lipids. [^{35}S]GTPγS was from Perkin-Elmer.

GST Pull-Down Experiments. HeLa cells were mock-transfected or transfected with plasmids encoding myc-tagged endophilin B1, myc-tagged endophilin A2, and vsv-g–tagged exchange factor for Arf6 (EFA6) as indicated by using Jet Pei reagent. After 24 h, cells were lysed in 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1% Triton-X100, 2 mM DTT, and a mixture of protease inhibitors (Roche Diagnostics), and centrifuged at 15,000 × *g* for 10 min at 4 °C. Supernatants were incubated with 0.25 mM GST constructs in the presence of 0.75% BSA and glutathione–Sepharose beads overnight at 4 °C.

For direct binding assay with purified recombinant proteins, 0.05–0.15 μM His fusion proteins containing various fragments of endophilin or EFA6 as indicated were incubated in 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 2 mM DTT, and a mixture of protease inhibitors with 0.25 μM GST constructs with or without 0.5 mM liposomes in the presence of 0.75% BSA and glutathione–Sepharose beads for 2 h 30 min at 4 °C.

Beads were washed, and bound proteins were eluted by using SDS sample buffer and separated on SDS/PAGE. The presence of EFA6 and endophilin in the eluate was detected by Western blotting by using the anti-tag antibodies.

Confocal Immunofluorescence Microscopy. HeLa or TRVb-1 cells plated on 11-mm round glass coverslips were transiently transfected with pcDNA3 or pEGFP-N1/C1 constructs by using the Jet-PEI transfection reagent (Polyplus Transfection) as described by the manufacturer. Unless otherwise stated, the cells were washed twice in PBS solution 24 h after transfection and then fixed in 3% paraformaldehyde and processed for immunofluorescence analysis as described previously (1). Confocal microscopy was carried out with a TCS-SP5 laser scanning confocal microscope (Leica Microsystems).

Preparation of Phospholipid Vesicles. Large unilamellar vesicles of asolectin were prepared as described elsewhere (2) and extruded through a 0.4- or 0.05-μM pore-size polycarbonate filter (Isopore; Millipore). Vesicles of defined composition (dioleyl-phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine at 35/35/30 weight ratio) were prepared as previously described previously (3) at the concentration of 2 mg/mL.

[^{35}S]GTPγS Binding Assay. MyrArf6 (2 μM) was incubated at 30 °C with [^{35}S]GTPγS (20 μM; ~2,000 cpm/pmol) in 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 100 mM KCl, with asolectin (2 mM) with or without (as indicated in the Fig. 1 legend) His-tagged EFA6A (0.5 μM) and GST-endophilin (2 μM). At the indicated times, samples of 25 μL were removed and measured for radioactivity as described previously (4).

Flotation Experiments. Proteins (1.5–4 μM) and liposomes (1 mM) were incubated in HK buffer (50 mM Hepes pH 7.2; 120 mM K-acetate) with DTT (1 mM) and $MgCl_2$ (0.5 mM) at room temperature for 20 min in a total volume of 150 μL. The suspension was adjusted to 30% sucrose by adding and mixing 100 μL of a 75% wt/vol sucrose solution in HK buffer. The resulting high-sucrose suspension was overlaid with 200 μL HK containing 25% wt/vol sucrose and 50 μL HK containing no sucrose. The sample was centrifuged at 55,000 rpm (240,000 × *g*) in a swinging rotor (TLS 55; Beckman Coulter) for 1 h. The bottom (250 μL), middle (150 μL), and top (50 μL) fractions were manually collected from the bottom by using a Hamilton syringe, and analyzed by SDS-PAGE before and after staining with SYPRO Orange (Molecular Probes). The presence of proteins was detected by Western blot.

Dynamic Light Scattering. Measurements were made by using a Dynapro MSX instrument (Protein Solutions) equipped with a Peltier temperature controller. A 15-μL solution of Hepes 50 mM, pH 7.2, K-acetate 120 mM, and $MgCl_2$ 1 mM containing His-N-BAR (2 μM), with or without asolectin liposomes (0.1 mg/mL) and GST-Sec7 (5 μM) as indicated in Fig. 3, was placed in a quartz cuvette. After equilibration at 25 °C, 10 autocorrelation functions of the scattered light were determined at the optimal laser intensity, each for 10 s. From multiexponential fits of the autocorrelation functions, an intensity graph was produced, and the average size of particles represented (Dynamic V 5.0 software; Protein Solutions).

EM. Samples containing His-N-BAR (2μM) were incubated for 20 min at room temperature in HKM buffer (50 mM Hepes pH 7.5; 100 mM KCl; 2 mM $MgCl_2$) with or without asolectin liposomes (0.1 mg/mL) and GST-Sec7 (5 μM). Samples were deposited on glow discharge carbon-coated grids and negatively stained with 1% aqueous uranyl acetate. They were observed with a JEOL 1400 transmission electron microscope. Acquisitions were made with a Morada digital camera (Olympus SIS).

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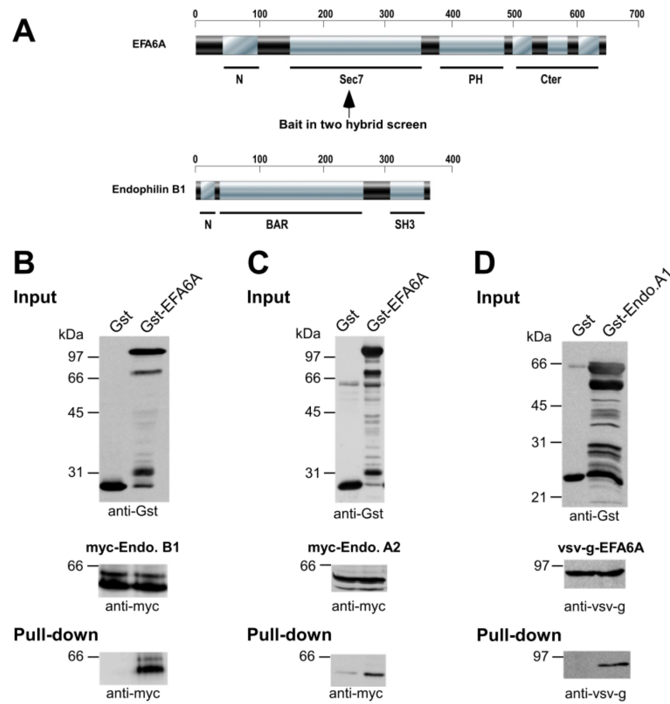

Fig. S1. Endophilin interacts with EFA6. (*A*) Schematic representation of the domain organization of EFA6A and endophilin B1. (*B* and *C*) GST pull-down of myc-tagged endophilin B1 (*B*) or A2 (*C*) expressed in HeLa cells by EFA6A fused to GST. (*D*) GST pull-down of vsv-g–tagged EFA6A expressed in BHK cells by endophilin A1 fused to GST. The results shown are representative of three independent experiments.

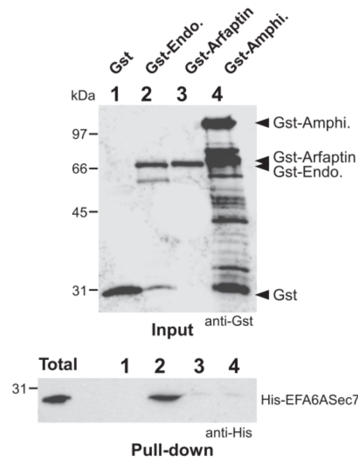

Fig. S2. EFA6A-Sec7 domain interacts specifically with GST-endophilin but not with GST-arfaptin 2, neither with GST-amphiphysin II. GST pull-down of purified His-tagged EFA6A-Sec7 domain using three BAR-domain containing proteins: endophilin A1, arfaptin 2, and amphiphysin II fused to GST. The results shown are representative of four independent experiments.

III. Résumé

Afin de confirmer les résultats obtenus en double hybride, nous avons réalisé des expériences de pull-down sur des lysats cellulaires sur-exprimant nos protéines d'intérêt. Nous avons pu observer qu'EFA6A interagit avec des isoformes représentants les deux familles d'endophiline, soit l'endophiline A1, l'endophiline A2 et l'endophiline B1. Nous avons pu mettre en évidence, par l'utilisation de protéines purifiées que cette interaction est directe et qu'elle se fait par l'intermédiaire du domaine Sec7 d'EFA6 et des 125 premiers acides aminés du domaine N-BAR de l'endophiline A1. Ces deux domaines étant importants pour les fonctions de ces deux protéines, nous avons ensuite étudié les effets de cette interaction sur leurs activités respectives. Nous avons observé qu'in vitro l'endophiline A1 stimule l'activité d'échange d'EFA6 sur de l'Arf6 myristylé en présence de vésicules de grande taille. Ces résultats suggèrent qu'EFA6 est capable de lier simultanément Arf6 et l'endophiline, et que par cette interaction l'endophiline joue un rôle de régulateur positif sur l'activité catalytique d'EFA6. Par des expériences de flottaison sur gradient de sucrose et de pull-down en présence de vésicules artificielles, nous avons mis en évidence une compétition entre le domaine Sec7 et les lipides pour la liaison au domaine N-BAR. Dans nos conditions expérimentales nous observons que l'interaction entre EFA6 et l'endophiline est contrôlée par la courbure membranaire. En effet, les expériences de pull down montrent que la présence de vésicules à fort degré de courbure dissocie le complexe EFA6/endophiline. Alors que le domaine N-BAR seul se retrouve dans la fraction Top liée aux vésicules mimant une membrane plane à la suite de l'expérience de flottaison, on le retrouve dans la fraction Bottom en présence du domaine Sec7. Ceci est en faveur d'un effet dissociateur du domaine Sec7 sur l'interaction entre le domaine N-BAR et les membranes planes. Ces résultats mettent en évidence pour la première fois le contrôle d'une interaction protéine-protéine par une courbure membranaire.

Nous avons pu observer par des expériences de diffusion dynamique de lumière et de microscopie électronique que l'endophiline A1 déforme et tubularise des vésicules de grande taille et que cet effet est inhibé en présence du domaine Sec7 d'EFA6.

Nous avons observé par des expériences d'immunofluorescence que l'endophiline A2 se localise principalement au niveau du cytosol alors qu'EFA6 se trouve à la membrane

plasmique au niveau de replis membranaires. Lorsque ces deux protéines sont co-transfectées, on observe que l'endophiline A2 se relocalise en partie à la membrane plasmique dans des structures riches en EFA6.

Nous nous sommes intéressés à l'internalisation du récepteur à la transferrine. En effet, des études précédentes menées au laboratoire avaient mis en évidence que la surexpression d'EFA6 inhibait l'internalisation de la transferrine et induisait la redistribution de son récepteur à la surface par un mécanisme inconnu. Dans cette étude, nous avons confirmé cet effet d'EFA6 et avons observé que la surexpression de l'endophiline A2 n'avait aucun effet sur l'internalisation de la transferrine. En revanche, nous observons dans les cellules surexprimant l'endophiline et EFA6, que l'internalisation de la transferrine se fait de manière similaire aux cellules contrôles et aux cellules surexprimant l'endophiline A2 seule. Ceci montre que la surexpression d'endophiline est capable de lever l'effet inhibiteur de la surexpression d'EFA6 sur l'internalisation de la transferrine. De plus, ces résultats indiquent que l'endophiline et EFA6 agissent sur la même voie pour contrôler l'internalisation de la transferrine. Pour expliquer ces résultats nous avons retenus l'hypothèse qu'EFA6 surexprimé piégerait l'endophiline endogène l'empêchant ainsi d'assurer ses fonctions dans l'endocytose et qu'une surexpression complémentaire de l'endophiline est nécessaire pour réverser cet effet.

IV. Résultats complémentaires

Dans le but d'approfondir les résultats obtenus sur l'effet stimulateur de l'endophiline A1 sur l'activité d'EFA6, nous avons réalisé des expériences complémentaires. Nous avons donc étudié l'effet de concentrations croissantes d'endophiline A1 sur la cinétique d'échange nucléotidique d'Arf6 catalysée par EFA6. Nous observons que plus la concentration d'endophiline A1 dans le milieu est importante, plus la liaison d'Arf6 au GTPγS est élevée. L'endophiline augmente l'activité d'échange d'EFA6 sur Arf6 de manière dépendante de la dose (Figure 34).

Au cours de cette étude nous avons également cherché à déterminer si l'interaction du domaine Sec7 d'EFA6 avec le domaine N-BAR est spécifique de l'endophiline. Pour cela, nous avons réalisé une expérience de pull-down avec le domaine Sec7 d'EFA6 et différentes protéines possédant des domaines N-BAR dont l'amphyphysine, l'Arfaptine et l'endophiline

en contrôle. Nous avons pu observer que ni l'amphiphysine, ni l'Arfaptine n'interagissaient directement avec le domaine Sec7 d'EFA6. Ces résultats suggèrent que la structure 3D n'est pas suffisante et que la séquence primaire du domaine BAR est importante pour la liaison à EFA6 (Figure article S2).

Nous nous sommes également intéressés au rôle de cette interaction sur l'internalisation d'autres récepteurs et notamment le récepteur β2-adrénergique. Nous savons que la fixation du ligand induit l'internalisation du RCPG activé. Afin de déterminer si l'endophiline et EFA6 ont un rôle sur cette internalisation, nous avons effectué des expériences d'immunofluorescence sur des cellules HEK exprimant stablement le récepteur β2-adrénergique couplé à la GFP et transfectées avec l'endophiline A2 seule ou avec EFA6. Suite à l'addition d'isoprotérenol, nous observons une internalisation du récepteur. Le récepteur s'accumule dans des vésicules à l'intérieur des cellules. Cette internalisation est dépendante du temps d'incubation avec le ligand. Nous pouvons observer que plus la durée d'incubation avec le ligand est longue, plus le récepteur se retrouve à l'intérieur des cellules. Nous n'observons aucune différence significative sur l'internalisation du récepteur β2-adrénergique quand l'endophiline A2 est transfectée seule ou avec EFA6 (Figure 35). Cette expérience ne nous permet donc pas de conclure quant au rôle de ces deux protéines et de leur interaction sur l'internalisation de ce récepteur, mais suggère que la quantité de ces deux protéines n'est pas limitante car leur surexpression n'accélère pas le processus d'internalisation. Dans le but de répondre à cette question, il serait intéressant d'éteindre l'expression de ces deux protéines dans les cellules à l'aide de siRNA. Cependant l'existence de plusieurs isoformes de ces deux protéines complique l'interprétation de ces expériences.

Des expériences menées au laboratoire ont montré que dans des cellules HEK transfectées stablement avec le récepteur β2-adrénergique couplé à la GFP et cultivées dans un milieu avec sérum, EFA6 était capable de redistribuer le récepteur à l'intérieur des cellules. Des expériences plus approfondies menées au laboratoire suggèrent que cet effet ne serait pas dû à une stimulation de l'internalisation mais à une inhibition du recyclage et serait dépendant de l'activation d'Arf6. Afin de visualiser la redistribution du récepteur β2 adrénergique induite par EFA6 et de déterminer si l'expression de l'endophiline affectait cet effet, nous avons réalisé des expériences d'immunofluorecence sur les cellules HEK exprimant stablement le récepteur β2-adrénergique et transfectées avec EFA6 et/ou l'endophiline A2. Nous observons dans les cellules surexprimant EFA6 et cultivées dans un

milieu sans sérum, que le récepteur β2-adrénergique se localise majoritairement à la membrane plasmique (Figure 36, panneau gauche). Cependant, lorsque ces cellules surexprimant EFA6 sont cultivées en présence de sérum, nous constatons une forte redistribution du récepteur à l'intérieur des cellules (Figure 36, panneau droite). En revanche, l'endophiline A2 ne semble pas avoir d'effet sur la localisation du récepteur que l'on soit en présence ou en absence de sérum. Cet effet du sérum dépend de la présence d'EFA6 car en absence d'EFA6 le récepteur se retrouve principalement à la membrane plasmique.

Nous observons aussi que l'expression de l'endophiline A2 n'inhibe pas la redistribution du récepteur induite par l'expression d'EFA6 en présence de sérum.

Ces résultats nous montrent qu'EFA6 est capable de redistribuer le récepteur en présence de sérum et que cette redistribution n'est pas affectée par la sur-expression d'endophiline. L'endophiline ne semble donc pas jouer un rôle dans le mécanisme de recyclage du récepteur β2-adrénergique stimulé ou non.

Nous nous sommes aussi intéressés à la dynamique des puits d'endocytose par la technique de TIR-FM (Total Internal Reflection Fluorescence Microscopy) autrement appelée microscopie à ondes évanescentes. Cette technique permet d'observer les protéines fluorescentes au voisinage de la membrane plasmique (à moins de 200nm). Elle est particulièrement adaptée pour visualiser les protéines présentes au niveau des puits et de déterminer leurs temps de résidence. Nous constatons ainsi que dans les cellules l'endophiline A2 a un marquage ponctiforme, suggérant une localisation au niveau des puits d'endocytose. Nous observons également que les points marqués par l'endophiline A2 sont dynamiques et ont une durée de vie d'une dizaine de secondes. En revanche nous remarquons qu'EFA6 possède un marquage diffus traduisant une localisation à la membrane plasmique comme décrit précédemment (Figure 37). Ces résultats mettent en évidence que l'endophiline A2 semblent être présente au niveau des puits d'endocytose alors qu'EFA6 n'y est pas concentrée.

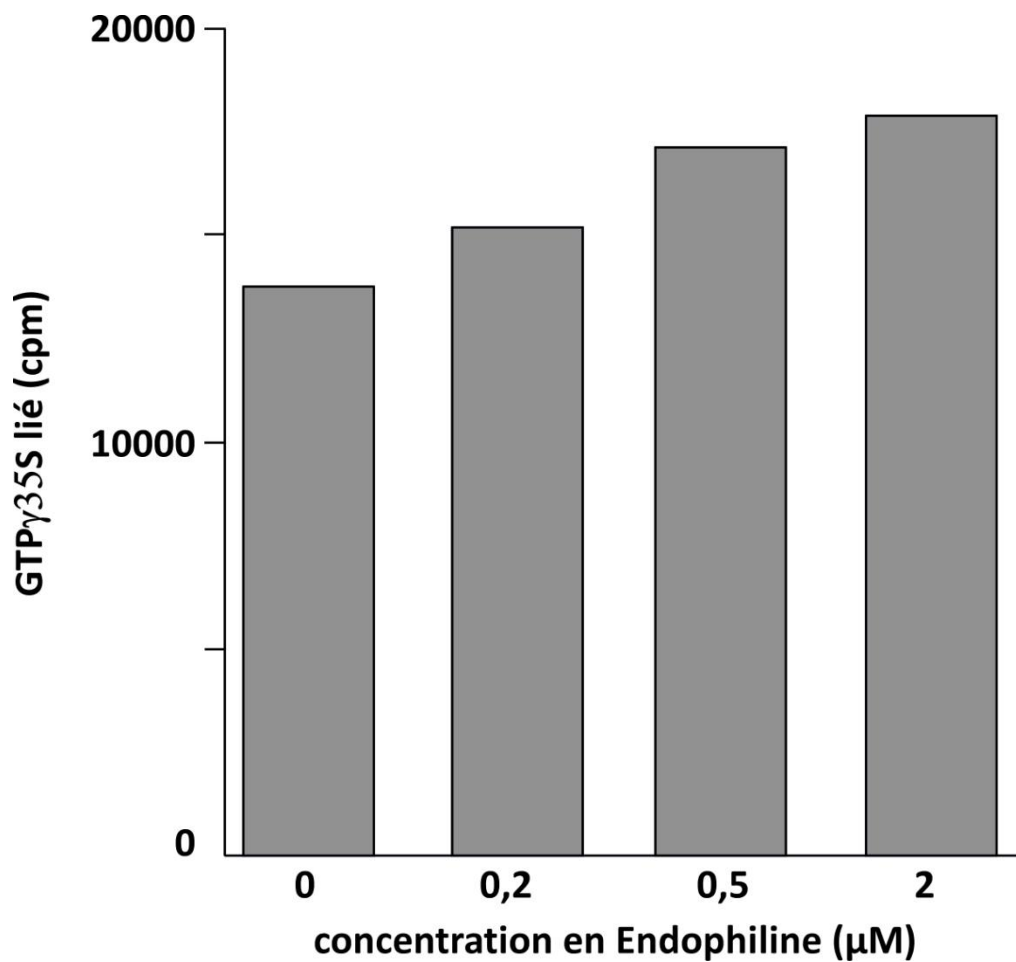

Figure 34: L'endophiline stimule l'activité catalytique d'EFA6 de manière dose dépendante.

La liaison du GTPγS[^{35}S] sur de l'Arf6 myristylé et purifié (2µM) a été mesurée en présence de vésicules artificielles, d'His-EFA6 (200nM) et de différentes concentrations de GST-endophiline A1 (0, 0.2, 0.5 et 2µM). La quantité d'Arf6 liée au GTPγS est déterminée au bout de 30min par filtration sur membrane de nitrocellulose et est exprimée en cpm (coups par minute)

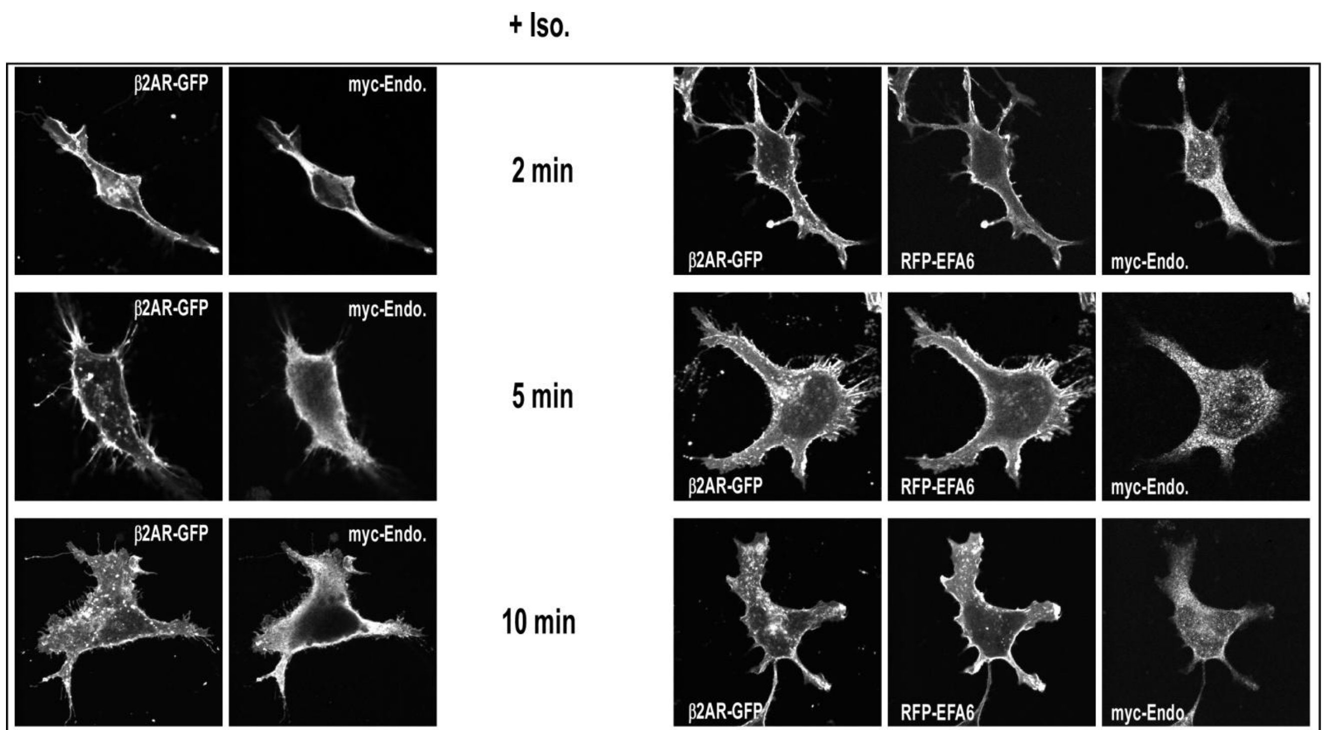

Figure 35: Le complexe EFA6/endophiline ne semble pas perturber l'internalisation du récepteur β2-adrénergique.

Les cellules HEK exprimant stablement le récepteur β2-adrénergique couplé à la GFP sont transfectées avec la myc-endophiline A2 +/-RFP-EFA6. 24h après transfection, les cellules sont maintenues dans un milieu sans sérum pendant 5h puis les récepteurs sont activés par 100nM d'isoprotérénol pendant 2, 5 ou 10 min. L'endophiline est révélée par un anticorps anti-myc (9E10).

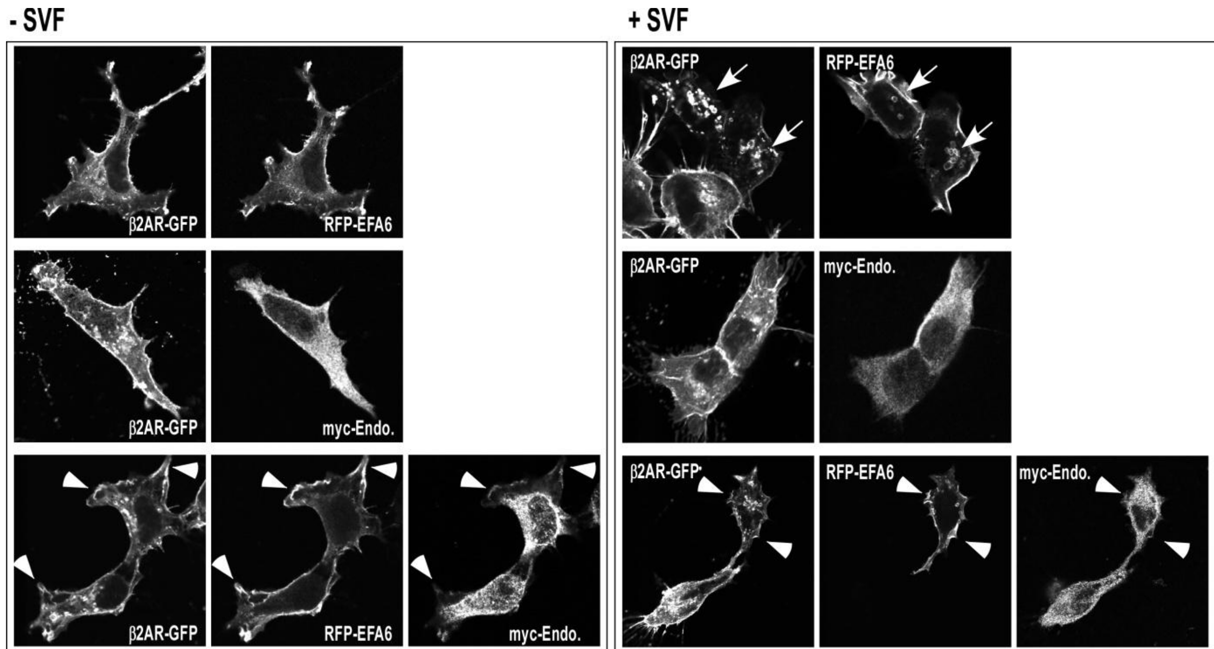

Figure 36: L'effet d'EFA6 sur la redistribution du récepteur β2-adrénergique n'est pas perturbé par son interaction avec l'endophiline.

Les cellules HEK exprimant stablement le récepteur β2-adrénergique couplé à la GFP sont transfectées avec la myc-endophiline A2 ou RFP-EFA6 seule ou ensemble. 24h après transfection les cellules sont maintenues dans un milieu sans ou avec sérum (respectivement –SVF et +SVF). L'endophiline est révélée par un anticorps anti-myc (9E10).

Les flèches montrent une colocalisation.

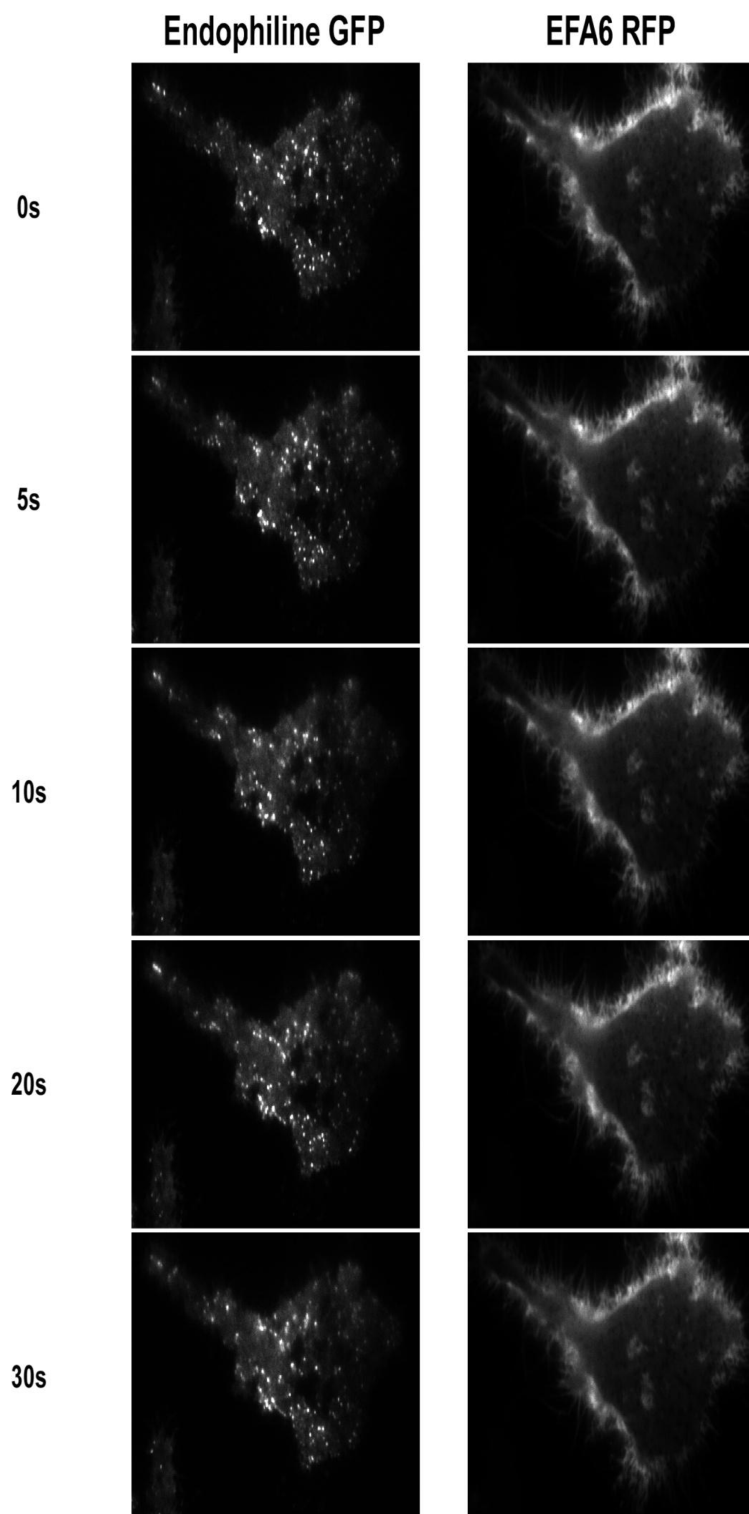

Figure 37 : L'endophiline se localise au niveau des puits d'endocytose lors de l'internalisation du récepteur β2-adrénergique

Les cellules Hela sont transfectées avec la GFP-endophiline A2, RFP-EFA6 et le récepteur β2-adrénergique HA. 48h après transfection les cellules incubées dans du PBS glucose (4g/l), sont observées en TIR-FM après stimulation des récepteurs par 1µM d'isoprotérenol.

V. Conclusion

Dans cette étude nous avons pu identifier et caractériser l'interaction directe entre le facteur d'échange EFA6 et l'endophiline. Cette interaction, qui à notre connaissance est la première décrite entre un facteur d'échange pour les Arf et une protéine à domaine Bar, renforce le lien entre la voie de signalisation EFA6/Arf6 dépendante et le trafic vésiculaire intracellulaire. L'ensemble des résultats obtenus nous a permis de proposer un modèle rendant compte du rôle d'EFA6 et de l'endophiline. En effet, EFA6 serait un des facteurs permettant le recrutement de l'endophiline à la membrane plasmique. Lorsque la membrane est plane, le complexe formé entre EFA6 et l'endophiline serait stable et permettrait l'activation d'Arf6. En revanche, au voisinage d'une vésicule en formation, le domaine N-BAR sensible à la courbure se dissocierait du domaine Sec7 d'EFA6 afin de se lier à la vésicule et assurer sa fonction.

En conclusion, cette étude met en évidence que l'endophiline et EFA6 agissent comme des régulateurs de l'internalisation de certains récepteurs et coopèrent pour réguler l'endocytose clathrine dépendante.