

Mécanismes de régulation d'EFA6

I. Introduction

Dans la seconde partie de ma thèse je me suis intéressée aux mécanismes de régulation du facteur d'échange EFA6. EFA6 est le premier facteur d'échange spécifique de la petite protéine G Arf6 à avoir été identifié. Il existe 4 isoformes d'EFA6 qui partagent la même structure. Celle-ci consiste en un domaine N-terminal très peu conservé et dont le rôle est encore indéterminé, un domaine Sec7 qui est responsable de l'activité catalytique de cette protéine, un domaine PH capable d'interagir avec les phospholipides permettant ainsi sa localisation à la membrane plasmique et un domaine C-terminal composé d'un domaine coiled-coil et de deux domaines riches en proline et responsable de la réorganisation du cytosquelette d'actine (Franco et al., 1999). Cet effet du C-terminal est probablement du en parti à son interaction directe avec l'actine filamenteuse (Macia et al., 2008) et l' α -actinine (Sakagami et al., 2007). EFA6 a été décrit comme étant un acteur dans différents processus cellulaires. Elle intervient dans la mise en place de la polarité épithéliale en favorisant et stabilisant l'assemblage des jonctions serrées (Klein et al., 2008; Luton et al., 2004). Comme nous avons pu le voir précédemment EFA6 est également impliqué dans le trafic intracellulaire de différentes protéines membranaires telles que le récepteur à la transferrine (Franco et al., 1999), le récepteur β 2-adrénergique (Macia et al., 2012) ou le canal potassique TWIK1 (Decressac et al., 2004). Nous avons mis en évidence qu'EFA6 interagit directement avec l'endophiline (Boulakirba et al., 2014), une protéine importante dans l'endocytose, et que cette interaction participe à l'endocytose dépendante de la clathrine. Malgré les différentes études sur les fonctions d'EFA6, son mécanisme de régulation reste encore inconnu. En effet, pour assurer spécifiquement ses fonctions EFA6 ne peut pas être constutivement actif mais doit l'être dans des conditions spatio-temporelles précises. Ainsi déterminer les mécanismes moléculaires qui contrôlent l'activation des facteurs d'échanges est essentiel pour comprendre l'ensemble des voies qu'ils régulent. Différentes études ont donc porté sur la détermination des mécanismes de régulation des facteurs d'échange et notamment du facteur d'échange ARNO (Hiester and Santy, 2013; Stalder et al., 2011). ARNO appartient lui aussi à la famille des facteurs d'échange à domaine

Sec7 et est impliqué dans la régulation des petites protéines G Arf lors des processus de migration et de changement de la morphologie cellulaire. Une étude menée par l'équipe du Dr Santy a mis en évidence un repli intramoléculaire au sein de la protéine ARNO (Hiester and Santy, 2013). Ce repli régule l'activité d'échange d'ARNO en contrôlant son association aux membranes phospholipidiques qui sont des cofacteurs pour l'activation des Arfs. En effet, ARNO a été décrit comme pouvant être sous une forme auto-inhibée, où le domaine C-terminal polybasique, en association avec la région linker entre le domaine Sec7 et le domaine PH sur laquelle il se replie, joue le rôle de pseudo-substrat et inhibe l'activité catalytique du domaine Sec7. Il a été mis en évidence que le domaine coiled-coil en N-terminal se replie sur le reste de la protéine jouant un rôle dans l'auto-inhibition d'ARNO. Ce facteur d'échange a besoin de deux signaux distincts pour se retrouver dans une conformation pleinement active. Le premier signal permet la levée de l'inhibition par le pseudo-substrat et peut se faire par différents mécanismes seuls ou combinés. Il peut se faire par phosphorylation de la Sérine 392 du domaine polybasique par la protéine PKC (Protein Kinase C). L'interaction de la petite protéine G Arf6 sous sa conformation activée avec le domaine PH d'ARNO permet le recrutement de ce dernier à la membrane plasmique et a été montré comme favorisant la dissociation du pseudo-substrat. Ce premier signal permet d'obtenir une forme intermédiaire. Le second signal permet d'interrompre l'interaction entre le domaine coiled-coil et le reste de la protéine et ainsi, obtenir une forme capable d'interagir avec les phospholipides de la membrane plasmique ce qui la rend totalement active. Cette étape nécessite la phosphorylation par la protéine Akt de la Thréonine 276 du domaine PH (Figure 38). Par analogie avec le facteur d'échange ARNO et dans le but de mieux comprendre le rôle d'EFA6 ainsi que la voie Arf6, nous nous sommes intéressés à ses mécanismes de régulation.

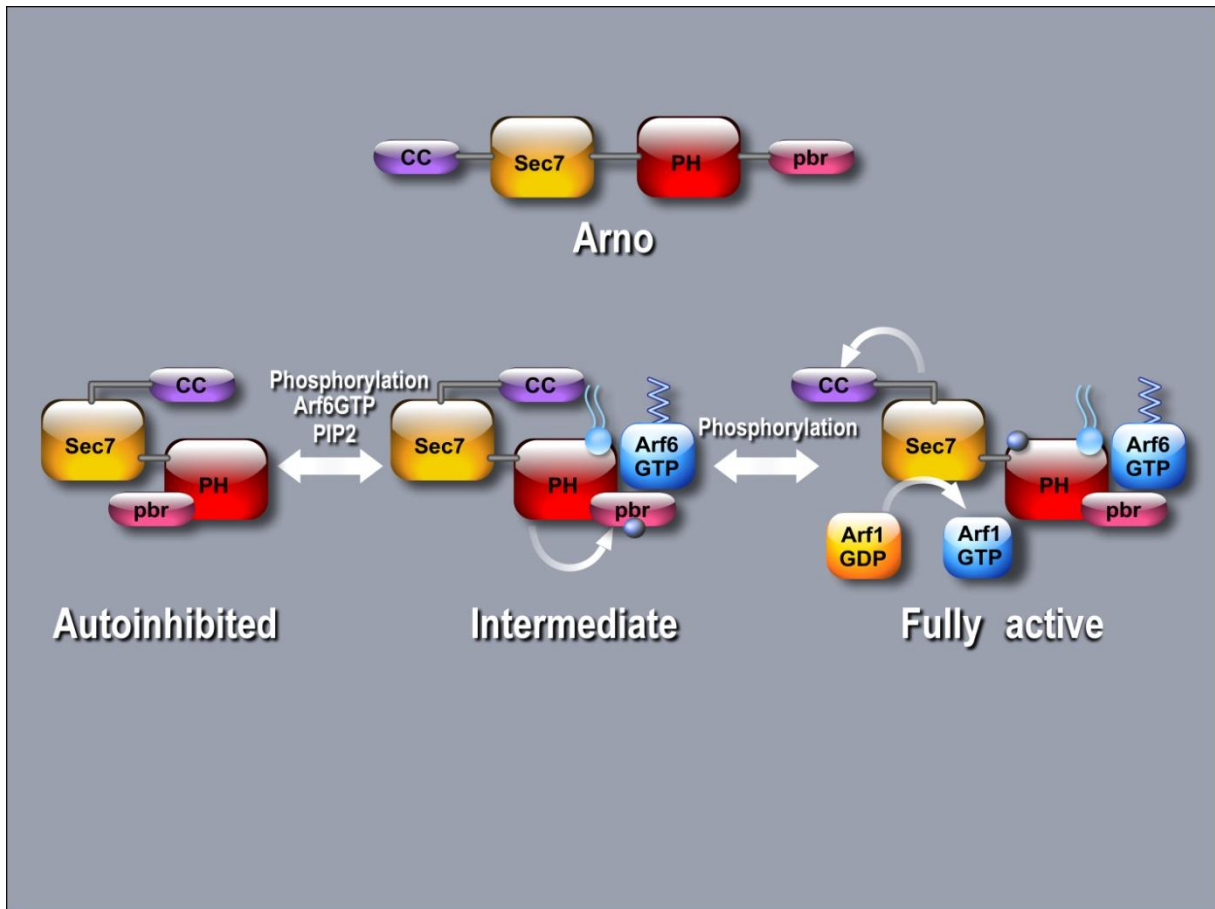


Figure 38: Schéma des mécanismes de régulation du facteur d'échange ARNO (Hiester and Santy, 2013).

Le passage d'un état inactif à un état activé du facteur d'échange ARNO est dépendant de son état de phosphorylation et de son interaction avec la protéine Arf6-GTP et les phospholipides.

II. EFA6 régule l'activation d'Arf1 et d'Arf6 par une boucle de rétrocontrôle négatif

A. Introduction

Les membres de la famille des facteurs d'échange à domaine Sec7, EFA6, BRAG et Cytohésine possèdent des domaines N-terminaux divergents mais partagent une organisation commune. En effet ils sont composés d'un domaine Sec7 qui stimule l'échange GDP/GTP, suivi d'un domaine PH capable de lier les phospholipides membranaires et de réguler différentes fonctions. Dans cet article en collaboration avec l'équipe du Dr Jacqueline Cherfils, nous nous sommes intéressés aux mécanismes de régulation du facteur d'échange EFA6 par son domaine PH-C-terminal. Pour cela des expériences reconstituant l'activité d'échange GDP/GTP d'EFA6 sur les protéines Arfs ont été réalisées sur des membranes. L'activité d'échange du domaine Sec7 d'EFA6 seul ou combiné à son domaine PH-C-terminal sur les protéines Arf6 myristilées ou non ou ne possédant pas l'hélice α en N-terminal ($\Delta 13$ Arf6) a été mesurée par la technique de fluorescence du tryptophane. En effet, le tryptophane est un des acides aminés aromatiques composant les protéines et contribuant à la fluorescence intrinsèque de ces dernières. La fluorescence du tryptophane est utilisée pour suivre le changement de conformation des protéines Arf. Sur Arf6 comme sur Arf1 deux tryptophanes ont été identifiés comme étant sensibles aux changements de conformation lors du cycle GDP/GTP et responsables d'une forte augmentation de la fluorescence intrinsèque que l'on observe.

B. Résultats

Dans un premier temps les expériences ont été réalisées en solution en présence du $\Delta 13$ Arf6, qui est une forme soluble d'Arf6 car dépourvue de l'hélice N-terminal, et du domaine Sec7 ou des domaines Sec7-PH-C-terminal. Nous avons pu observer une faible activation du $\Delta 13$ Arf6 par le domaine Sec7. En revanche nous constatons que la cinétique d'échange est augmentée par un facteur 7 par la construction Sec7-PH-C-terminal. Ces

expériences ont également été réalisées en présence de liposomes contenant de PIP2 sur de l'Arf6 myristylée (myr-Arf6). Dans ces conditions, on remarque également que la présence de liposomes stimule la cinétique d'activation de la protéine myrArf6 catalysée par le Sec7 et que cette stimulation est plus importante en présence du Sec7-PH-C-terminal.

Ces résultats mettent en évidence que, contrairement à ARNO, le domaine PH-C-terminal d'EFA6 n'est pas auto-inhibiteur sur son activité d'échange et que cette dernière est stimulée en présence de liposomes. La composition des liposomes joue un rôle important dans cet effet activateur. En effet des expériences complémentaires ont permis de démontrer que comparativement aux expériences réalisées en solution l'ajout de liposomes non chargés n'avait pas d'effet sur la cinétique d'activation d'Arf6. En revanche nous avons pu observer que l'ajout de vésicules composées de PIP2, de PhosphatidylSérine (PS) ou des deux potentialise fortement l'échange nucléotidique catalysé par EFA6.

L'ensemble de ces résultats suggère que les protéines myrArf6 et Sec7-PH-C-terminal, capables d'interagir avec les phospholipides membranaires et notamment le PIP2, vont se retrouver colocalisées, avec une orientation optimale, au niveau des membranes. Cette réduction de dimensionnalité entre EFA6 et son substrat potentialiserait ainsi l'activation d'Arf6.

De façon intéressante, cette étude a également montré qu'Arf6-GTP, le produit de la réaction catalysée par EFA6, joue un rôle dans le rétrocontrôle négatif de son facteur d'échange. En effet, on constate qu'en présence d'Arf6-GTP incubée préalablement avec les liposomes, l'effet catalytique d'EFA6 sur l'échange nucléotidique est inhibé. De plus cette inhibition est dose dépendante et est spécifique de l'isoforme Arf6.

La suite du projet a porté sur la détermination du mécanisme par lequel Arf6-GTP induit son effet inhibiteur. Des expériences réalisées en solution ont montré que le $\Delta 13$ Arf6-GTP n'a pas d'effet sur l'activation de $\Delta 13$ Arf6-GDP par le Sec7 montrant qu'Arf6-GTP n'agit pas par une compétition pour la liaison du domaine Sec7. Nous avons donc émis l'hypothèse que l'effet d'Arf6-GTP était dû à une interaction avec le domaine PH-C-terminal. Pour tester cette hypothèse des liposomes ont été incubés préalablement avec de l'Arf6-GTP puis des doses croissantes de PH-C-terminal purifié ont été ajoutées à ce mélange avant le début de la réaction catalysée par EFA6 Sec7-PH-C-terminal. Nous avons pu observer une diminution de

l'inhibition induite par Arf6-GTP en présence du domaine PH-C-terminal. Nous remarquons également que plus la concentration de PH-C-terminal est importante plus l'inhibition induite par Arf6 est diminuée. Cette expérience met en évidence que le domaine PH-C-terminal est capable, de manière dose dépendante, de bloquer le rétrocontrôle négatif induit par Arf6-GTP. Ces résultats suggèrent aussi que le domaine PH-C-terminal lève cet effet inhibiteur en séquestrant l'Arf6-GTP.

Mon rôle dans ce projet a été de confirmer cette interaction entre le domaine PH-C-terminal d'EFA6 et Arf6-GTP. Pour cela j'ai réalisé une expérience de pull-down sur des lysats cellulaires surexprimant soit ArfQ67L soit Arf6T157N, deux formes liant principalement le GTP. Ainsi on a pu mettre en évidence que le domaine PH-C-terminal est capable d'interagir avec ces deux constructions d'Arf6 liées au GTP. Cette interaction a été confirmée par une expérience de co-sédimentation que j'ai réalisée à l'aide de domaine PH-C-terminal d'EFA6 et d'Arf6-GTP non myristylée. Nous avons ainsi pu mettre en évidence que le domaine PH-C-terminal d'EFA6, qui est principalement lié aux membranes, est capable de recruter Arf6-GTP non myristylé, qui est principalement soluble, au niveau de liposomes. Cette expérience a ensuite été reproduite et confirmée par l'équipe du Dr Cherfils avec la protéine $\Delta 13$ Arf6-GTP.

L'ensemble de ces résultats a permis de proposer un modèle selon lequel EFA6 serait régulé par un rétrocontrôle négatif induit par l'interaction de son produit Arf6-GTP avec son domaine PH-C-terminal.



EFA6 controls Arf1 and Arf6 activation through a negative feedback loop

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Guanine nucleotide exchange factors (GEFs) of the exchange factor for Arf6 (EFA6), brefeldin A-resistant Arf guanine nucleotide exchange factor (BRAG), and cytohesin subfamilies activate small GTPases of the Arf family in endocytic events. These ArfGEFs carry a pleckstrin homology (PH) domain in tandem with their catalytic Sec7 domain, which is autoinhibitory and supports a positive feedback loop in cytohesins but not in BRAGs, and has an as-yet unknown role in EFA6 regulation. In this study, we analyzed how EFA6A is regulated by its PH and C terminus (Ct) domains by reconstituting its GDP/GTP exchange activity on membranes. We found that EFA6 has a previously unappreciated high efficiency toward Arf1 on membranes and that, similar to BRAGs, its PH domain is not autoinhibitory and strongly potentiates nucleotide exchange on anionic liposomes. However, in striking contrast to both cytohesins and BRAGs, EFA6 is regulated by a negative feedback loop, which is mediated by an allosteric interaction of Arf6-GTP with the PH-Ct domain of EFA6 and monitors the activation of Arf1 and Arf6 differentially. These observations reveal that EFA6, BRAG, and cytohesins have unanticipated commonalities associated with divergent regulatory regimes. An important implication is that EFA6 and cytohesins may combine in a mixed negative-positive feedback loop. By allowing EFA6 to sustain a pool of dormant Arf6-GTP, such a circuit would fulfill the absolute requirement of cytohesins for activation by Arf-GTP before amplification of their GEF activity by their positive feedback loop.

endocytosis | membrane traffic | kinetics | autoinhibition

Guanine nucleotide exchange factors (GEFs), which activate small GTPases by stimulating their intrinsically very slow GDP/GTP exchange, are key players in the extraordinary diversity of small GTPases pathways (reviewed in ref. 1). Small GTPases carry little specificity determinants on their own to determine when and where they should be turned on and which pathway they should activate (2), which are instead largely monitored by their GEFs. Thus, understanding how different members of a GEF family activate an individual small GTPase in distinct patterns is a major issue in small GTPase biology in normal cells and in diseases.

An important contribution to the functional specificity of GEFs is how they themselves are regulated. Crystallographic studies combined with biochemical studies that reconstituted GEF-stimulated GDP/GTP nucleotide exchange have been instrumental in uncovering a growing complexity of regulatory mechanisms (reviewed in ref. 1). These include autoinhibitory elements outside the catalytic GEF domain that block access to the active site (3–7), large conformational changes that release autoinhibition in response to various stimuli (8–11), positive feedback loops in which freshly produced GTP-bound GTPases stimulate GDP/GTP exchange (10, 12–15), and potentiation of nucleotide exchange by colocalization on membranes (11, 13, 16, 17).

These previous studies demonstrated that a wide range of regulatory regimes can be achieved even at the scale of a single GEF family by regulatory mechanisms that combine in multiple ways.

GEFs that activate small GTPases of the Arf family (ArfGEFs), which are major regulators of many aspects of membrane traffic and organelle structure in eukaryotic cells (reviewed in refs. 18 and 19), form one of the best-characterized GEF families to date (reviewed in ref. 1), making a comprehensive view of their regulatory repertoire within reach. ArfGEFs comprise two major groups: the BIG/GBF1 group, which functions at the Golgi, and a group composed of the exchange factor for Arf6 (EFA6), brefeldin A-resistant Arf guanine nucleotide exchange factor (BRAG), and cytohesin subfamilies, which activate Arf GTPases at the cell periphery and function in various aspects of endocytosis (reviewed in ref. 20). The actual substrates of these ArfGEFs have been difficult to establish, notably because the most abundant Arf isoform, Arf1, was long believed to be excluded from the plasma membrane where the Arf6 isoform is located. Accordingly, cytohesins and BRAGs have been described as Arf6-specific GEFs in cells but are now recognized as active Arf1-GEFs (16, 21, 22), whereas EFA6 remains the sole ArfGEF considered to be strictly Arf6-specific (23, 24).

Members of the EFA6, BRAG, and cytohesin subfamilies have divergent N-terminal domains but a related domain organization in their C terminus comprising a Sec7 domain, which stimulates GDP/GTP exchange, followed by a pleckstrin homology (PH) domain, which has multiple regulatory functions. In cytohesins, the PH domain recognizes signaling phosphoinositides by its canonical lipid-binding site (25), autoinhibits the Sec7 domain by obstructing its Arf-binding site (4), and amplifies nucleotide

Significance

EFA6, cytohesins, and BRAGs activate Arf GTPases in endocytic events. They carry a plasma membrane-binding PH domain in tandem with their catalytic Sec7 domain, which is autoinhibitory and mediates a positive feedback loop in cytohesins but not in BRAGs, and has an as-yet unknown role in EFA6 regulation. By reconstituting GDP/GTP exchange on membranes, we find that the PH domain of EFA6 is not autoinhibitory, but supports a negative feedback loop. This loop is controlled by interaction of Arf6-GTP with the PH-Ct domains of EFA6 and monitors Arf1 and Arf6 activation differentially. This suggests that EFA6 and cytohesins might be coupled in a mixed negative-positive feedback loop to shape the level and timing of Arf1 and Arf6 activation in endocytosis.

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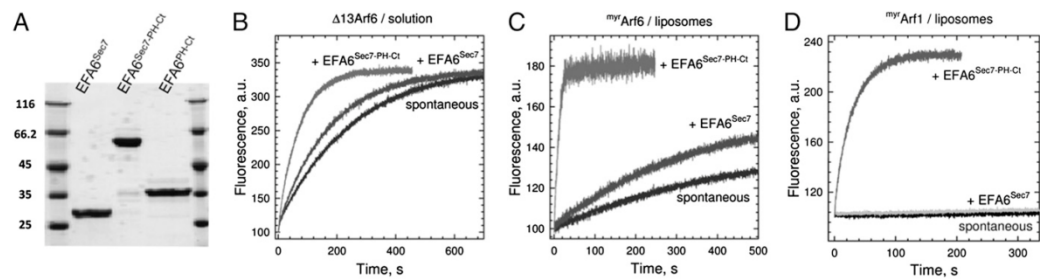


Fig. 1. Regulation of Arf1 and Arf6 activation by EFA6 on membranes. (A) EFA6 constructs used in this study (12% SDS/PAGE, 3 μ g/lane). (B) EFA6 is not autoinhibited by its PH-Ct domain. Representative tryptophan fluorescence kinetic traces for Δ 13Arf6 (1 μ M) activation in solution by EFA6 constructs (100 nM) as indicated. (C) EFA6 is strongly potentiated by membranes. Representative kinetic traces for myr Arf6 (0.4 μ M) activation by EFA6^{Sec7-PH-Ct} (10 nM) or EFA6^{Sec7} (220 nM) in the presence of liposomes. (D) EFA6 is a potent Arf1GEF on membranes. Representative tryptophan fluorescence kinetic traces for myr Arf1 activation by EFA6^{Sec7-PH-Ct} (3.75 nM) or EFA6^{Sec7} (575 nM).

exchange by a positive feedback loop involving its direct interaction with Arf1-GTP or Arf6-GTP (10, 13, 21). In contrast, the PH domain of BRAG is not autoinhibitory and is not involved in a feedback loop, but instead strongly potentiates nucleotide exchange by binding to polyanionic membranes without marked phosphoinositides preference (16).

How members of the EFA6 subfamily are regulated is currently unknown. These ArfGEFs are found predominantly (although not exclusively) in the brain and function in the coordination of endocytosis and actin dynamics (23, 26, 27), in the maintenance of tight junctions (28), in microtubule dynamics in *Caenorhabditis elegans* embryos (29), and in the formation and maintenance of dendrites (30), although the molecular details of these functions remain largely unknown. Consistent with an important role in the brain, defects in EFA6 functions have been found in neurologic disorders (31) and in human gliomas (32). The PH domain of EFA6 subfamily members drives the localization of EFA6 members to the plasma membrane (26) and it binds to PIP₂ lipids (33). It is followed by a 150-residue C-terminal (Ct) domain predicted to form a coiled coil, which massively induces actin-rich membrane protrusions when expressed with the PH domain (26). The divergence of regulatory mechanisms between Sec7-PH-containing cytohesins and BRAGs prompted us to undertake a quantitative biochemical investigation of EFA6 nucleotide exchange regulation. Our findings reveal an overlooked dual specificity of EFA6 for Arf1 and Arf6 and an unprecedented regulation by a negative feedback loop, with important potential implications for the activation of Arf GTPases in endocytic events.

Results

EFA6 Is Not Autoinhibited by Its PH Domain and Is Potentiated by Membranes. To quantify the GEF efficiency of human EFA6, we performed nucleotide exchange kinetics analyses with EFA6A constructs encompassing the Sec7 domain (EFA6^{Sec7}) or the entire C-terminal region (EFA6^{Sec7-PH-Ct}) along with the cellular form of Arf6 that carries a myristate attached to its N-terminal glycine (myr Arf6), full-length Arf6 lacking the myristate group (unmyr Arf6), or a soluble construct lacking the N-terminal α -helix (Δ 13Arf6). All recombinant proteins were highly pure (Fig. 1A and Fig. S1; for Arf proteins, see refs. 16 and 34), allowing accurate kinetics analysis using tryptophan fluorescence (Table 1).

We first analyzed the nucleotide exchange efficiency (k_{cat}/K_M) of the different EFA6 constructs in solution (Fig. 1B). EFA6^{Sec7} activated Δ 13Arf6 with $k_{cat}/K_M = 1.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, which is in the same range as values reported for cytohesins (35) and BRAGs (16). EFA6^{Sec7-PH-Ct} was more active than EFA6^{Sec7}

by approximately sevenfold, indicating that the PH-Ct domain is not autoinhibitory but instead contributes a slight potentiation. EFA6^{Sec7} and EFA6^{Sec7-PH-Ct} were essentially inactive toward unmyr Arf6 (in the $10^3 \text{ M}^{-1}\text{s}^{-1}$ range), demonstrating that neither the Sec7 domain nor the PH-Ct domain carry determinants to displace the N-terminal helix of Arf6, which locks its GDP-bound form (36).

We next analyzed the efficiency of our EFA6 constructs in the presence of PIP₂-containing anionic liposomes (Fig. 1C). EFA6^{Sec7} was insensitive to liposomes, whereas liposomes increased the catalytic efficiency of EFA6^{Sec7-PH-Ct} toward unmyr Arf6, which tethers weakly to membranes by its amphipathic N-terminal helix, but not toward Δ 13Arf6, which lacks all membrane-tethering elements. The maximal k_{cat}/K_M value ($2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) was reached for myr Arf6, in which all membrane-tethering elements are present, which is approximately three orders of magnitude more efficient than EFA6^{Sec7}/ Δ 13Arf6 in solution. These observations indicate that a major component of EFA6 full efficiency stems from its recruitment to membranes by its PH-Ct domain.

We next analyzed whether the efficiency of EFA6 would be sensitive to liposome characteristics, as has been shown recently for other human and bacterial ArfGEFs (11, 13, 16) (Table S1 and Fig. S24). The addition of uncharged liposomes had essentially no effect compared with experiments carried out in solution, whereas all liposomes containing PIP₂, PS, or both strongly potentiated nucleotide exchange. Surprisingly, the effects of PIP₂ and PS were not additive, raising the possibility that EFA6 could bind to membranes by unspecific electrostatic interactions, as was recently reported for BRAG (16). EFA6^{Sec7-PH-Ct} and EFA6^{PH-Ct}

Table 1. Catalytic efficiencies (k_{cat}/K_M , $\text{M}^{-1}\text{s}^{-1}$) of EFA6^{Sec7} or EFA6^{Sec7-PH-Ct} toward Arf1 and Arf6 constructs in the absence (–) or presence (+) of 100 μ M liposomes

Substrate		EFA6 ^{Sec7}	EFA6 ^{Sec7-PH-Ct}
Δ 13Arf6	–	$1.70 \pm 0.02 \cdot 10^4$	$1.11 \pm 0.02 \cdot 10^5$
	+	ND	$2.90 \pm 0.07 \cdot 10^5$
unmyr Arf6	–	$6.87 \pm 0.24 \cdot 10^2$	$1.13 \pm 0.07 \cdot 10^3$
	+	$7.80 \pm 0.38 \cdot 10^2$	$1.42 \pm 0.05 \cdot 10^6$
myr Arf6	+	$5.07 \pm 0.17 \cdot 10^3$	$2.06 \pm 0.08 \cdot 10^7$
Δ 17Arf1	–	$3.18 \pm 0.12 \cdot 10^2$	$1.13 \pm 0.05 \cdot 10^3$
unmyr Arf1	–	ND	$1.67 \pm 0.35 \cdot 10^2$
	+	$5.36 \pm 0.55 \cdot 10^2$	$5.61 \pm 0.13 \cdot 10^6$

ND, not determined.

All values are the average \pm SD of three independent experiments.

bound IP₃, the inositol-phosphate headgroup of PIP₂, with K_D values in the same range as those reported for GST-EFA6^{PH} and PIP₂-containing vesicles (33) (Fig. S2 B and C), suggesting that EFA6 interacts with PIP₂ in a specific manner. The addition of IP₃ did not have a significant effect on the nucleotide exchange efficiency of EFA6^{Sec7-PH-Ct} in solution (Fig. S2D), suggesting that binding of PIP₂ to the PH domain does not exert an allosteric effect on the Sec7 domain. Finally, EFA6 did not appear to be sensitive to the curvature of liposomes (Fig. S2E) or to the presence of cholesterol (Table S1).

Taken together, the foregoing experiments suggest that membranes potentiate EFA6^{Sec7-PH-Ct} efficiency through colocalization with ^{myr}Arf6 and the resulting optimization of their relative orientations, and that the PH-Ct domain has specific interactions with PIP₂ and possibly nonspecific electrostatic interactions with anionic lipids.

EFA6A Is an Efficient Arf1-GEF on Membranes. Previous studies found that EFA6 is inactive toward Arf1 in vitro (23, 26, 37), consistent with the long-prevailing view that Arf1 is excluded from the plasma membrane. However, more recent studies established that Arf1 is activated at the plasma membrane (21, 38) and showed that both Arf1 and Arf6 are substrates for cytohesins (4, 13) and BRAGs (16, 22). This prompted us to analyze the exchange efficiency of EFA6^{Sec7} and EFA6^{Sec7-PH-Ct} toward ^{myr}Arf1, ^{unmyr}Arf1, and Δ 17Arf1 in solution and on liposomes of varying composition (Table 1 and Table S1). EFA6 did not activate Arf1 in most cases (k_{cat}/K_M in the $10^3 \text{ M}^{-1}\text{s}^{-1}$ range or less), confirming previous observations. However, when using EFA6 and Arf1 constructs competent for membrane binding (EFA6^{Sec7-PH-Ct} and ^{myr}Arf1) in the presence of liposomes (Fig. 1D), k_{cat}/K_M increased by more than 10,000-fold compared with Δ 17Arf1/EFA6^{Sec7} in solution, reaching a catalytic efficiency of $5.6 \pm 0.1 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$, which is only 3.6 lower than the catalytic efficiency toward ^{myr}Arf6 on liposomes.

The insensitivity of Arf1 to activation by EFA6 in solution could be related to its switch 1 region, which is important for its interaction with ArfGEFs and has the same sequence in Arf1 and Arf6, but is structurally less dynamic in Arf1 (39) than in Arf6 (40). The Sec7 domain of EFA6 features an arginine residue (R625) that replaces a highly conserved valine that contacts the switch 1 of Arf1 in cytohesins (41), and thus would be well suited to sense this difference; however, the R625V mutation only slightly impaired Δ 13Arf6 activation (Fig. S3A), and it did not restore activation of Δ 17Arf1 by EFA6^{Sec7} (Fig. S3B). Thus, EFA6 is an efficient GEF for ^{myr}Arf1 on liposomes, but why

nucleotide exchange is not recapitulated with Δ 17Arf1 in solution cannot be explained by a simple sequence difference.

EFA6A Is Regulated by a Negative Feedback Loop Mediated by Arf6-GTP, but Not by Arf1-GTP. Positive feedback loops by freshly produced GTP-bound GTPases is emerging as a common trait of GEFs regulation (10, 12–15), although several examples have now been described that depart from this paradigm (7, 11, 16). To investigate the existence of a feedback mechanism in EFA6, we generated increasing amounts of ^{myr}Arf-GTP on liposomes before measuring nucleotide exchange, using all four possible combinations between Arf1 and Arf6. In striking contrast with the positive feedback effects observed in other GEFs, preloading liposomes with ^{myr}Arf6-GTP resulted in a significant decrease of nucleotide exchange from both ^{myr}Arf1 (Fig. 2 A and B) and ^{myr}Arf6 (Fig. S4A). ^{myr}Arf6-GTP inhibited ^{myr}Arf1 activation, with an IC₅₀ of $81 \pm 16 \text{ nM}$, and ^{myr}Arf6 activation, with an IC₅₀ of $944 \pm 203 \text{ nM}$ (Fig. 2C). In contrast, ^{myr}Arf1-GTP had no effect on EFA6 exchange rate, regardless of whether the substrate was ^{myr}Arf1 (Fig. 2C and Fig. S4B) or ^{myr}Arf6 (Fig. S4C). These results identify a negative feedback loop never reported before for a GEF, and they suggest that this loop discriminates between the two Arf isoforms.

We next investigated the mechanism through which Arf6-GTP mediates its negative feedback effect. A simple possibility could be that Arf6-GTP competes with Arf-GDP as an inhibitor or a substrate of the Sec7 active site. The addition of increasing concentrations of Δ 13Arf6-GTP had no effect on the activation of Δ 13Arf6 by EFA6^{Sec7} in solution, indicating that Arf6-GTP does not act through a simple competition effect (Fig. S5A). Likewise, EFA6^{Sec7-PH-Ct} was unable to stimulate GTP-GDP exchange from ^{myr}Arf6-GTP on liposomes (Fig. S5B), indicating that Arf6-GTP is not a substrate of EFA6.

Alternatively, ^{myr}Arf6-GTP could act through an allosteric interaction with the PH-Ct domain. To test this hypothesis, we added increasing amounts of purified EFA6^{PH-Ct} to liposomes preloaded with a fixed amount of ^{myr}Arf6-GTP before starting the exchange reaction with EFA6^{Sec7-PH-Ct} and ^{myr}Arf1. EFA6^{PH-Ct} relieved the feedback effect in a dose-dependent manner (Fig. 3A and Fig. S5C), suggesting that EFA6^{PH-Ct} reverses the negative feedback loop by titrating ^{myr}Arf6-GTP. Confirming that EFA6^{PH-Ct} binds to Arf6-GTP, Arf6Q67L and Arf6T157N expressed in cells, both loaded predominantly with GTP (42), were efficiently pulled down by purified GST-EFA6^{PH-Ct} (Fig. 3B). Finally, purified EFA6^{PH-Ct} was able to recruit Δ 13Arf6-GTP (which is soluble and does not bind to membranes) to

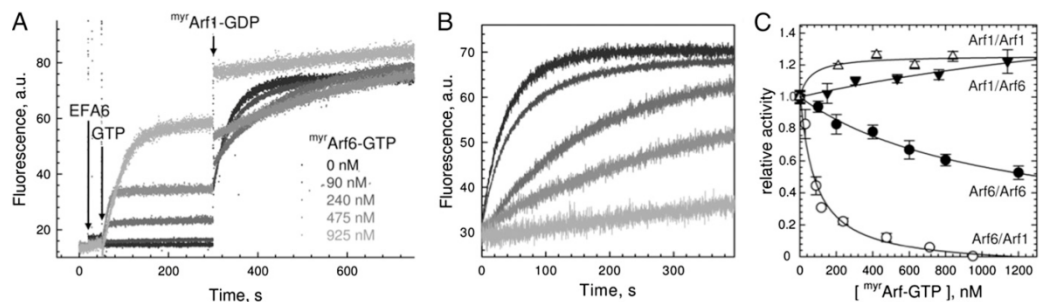


Fig. 2. EFA6A is regulated by a negative feedback loop mediated by Arf6-GTP. (A) A representative feedback loop experiment. Increasing amounts of ^{myr}Arf6-GTP (as indicated) were first generated on liposomes by EFA6^{Sec7-PH-Ct} (6 nM). After the plateau was reached, a second exchange reaction was initiated by the addition of 0.4 μM ^{myr}Arf1-GDP, from which k_{obs} values were determined. (B) Alignment of the second part of the reaction shown in A. (C) Relative activities for the different Arf1/Arf6 combinations as a function of the initial ^{myr}Arf-GTP amount generated on liposomes.

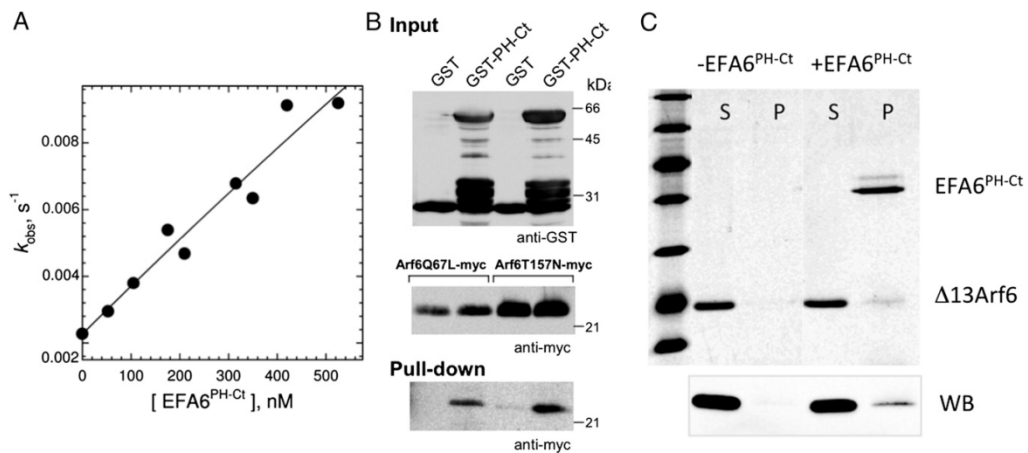


Fig. 3. The negative feedback effect is mediated by interaction of Arf6-GTP with EFA6^{PH-Ct}. (A) EFA6^{PH-Ct} reverses the negative feedback effect in a dose-dependent manner. ¹²⁵I-Arf1 activation was performed as in Fig. 2A, except with increasing amounts of EFA6^{PH-Ct} added before the first exchange reaction (fixed ¹²⁵I-Arf6-GDP concentration of 400 nM). (B) Pull-down of myc-tagged GTP-bound Arf6 mutants from cellular extracts by purified GST-tagged EFA6^{PH-Ct}. (C) Cosedimentation of Δ13Arf6-GTP with liposome-bound EFA6^{PH-Ct} analyzed by SDS/PAGE stained with Sypro orange (Upper) and by Western blot analysis with an anti-Arf antibody (Lower). S, supernatant; P, pellet.

liposomes (Fig. 3C), indicating that Arf6-GTP and EFA6^{PH-Ct} interact directly and independently of the catalytic Sec7 domain.

This ensemble of experiments suggests that the GEF activity of EFA6 is regulated by a negative feedback loop actuated specifically by ¹²⁵I-Arf6-GTP, and that the underlying mechanism involves an allosteric interaction of ¹²⁵I-Arf6-GTP with the PH-Ct domain of EFA6.

Discussion

Higher eukaryotes have three subfamilies of ArfGEFs involved in endocytic events—cytohesins, BRAGs, and EFA6—all of which carry a PH domain associated with a catalytic Sec7 domain. Previous studies highlighted that this organization determines different regulatory mechanisms in cytohesins (4, 10, 13, 21) and in BRAGs (16), but how the GEF activity of EFA6 is regulated by its PH domain remained unknown. By analyzing the regulation of EFA6A activity toward Arf1 and Arf6 using nucleotide exchange reactions reconstituted on artificial membranes, this study reveals unexpected commonalities and divergences among the three ArfGEF subfamilies, yielding a framework for envisioning how they work on peripheral membranes.

Our analysis shows that the Sec7 domain of EFA6 has a GEF efficiency toward Arf6 in the same range as that of cytohesins (35) and BRAGs (16). Likewise, the C-terminal region of EFA6, which includes the PH domain, supports strong potentiation of nucleotide exchange by membranes (by approximately three orders of magnitude), which is in the range reported for BRAG^{Sec7-PH} (16). Our study also reveals that EFA6^{Sec7-PH-Ct} is almost as potent an activator of Arf1 as Arf6 on membranes, possibly explaining why EFA6 leads to Arf1 activation in cell cultures (21). Taken

together, these observations reveal that EFA6 do not have major discrepancies with cytohesins and BRAGs in terms of intrinsic GEF efficiency or substrate preference.

In contrast, the regulatory modalities of EFA6 depart from those of cytohesins and BRAGs in various respects (Table 2). Notably, EFA6 is not autoinhibited by its PH domain and is regulated by a negative feedback loop mediated by interaction of its PH-Ct domains with Arf6-GTP. This interaction is reminiscent of the mechanism of feedback control of cytohesins by interaction of their PH domain with Arf-GTP (4, 10, 13, 21); however, the presence of a large C-terminal domain, which likely associates with the PH domain in EFA6 as suggested by small-angle X-ray scattering (SAXS) analysis (Fig. S1B), and the opposite regulatory effect of Arf-GTP suggest that the interaction probably is structurally very different. Furthermore, EFA6 discriminates between Arf1 and Arf6 as effectors of the negative feedback loop, an effect not seen in cytohesins, which are equally sensitive to activation by Arf1-GTP and Arf6-GTP (13). It should also be noted that EFA6 is potentiated equally well by PIP₂-containing or anionic membranes but does not have a strict requirement for a specific phosphoinositide, and thus has membrane preferences distinct from those of both cytohesins (25) and BRAGs (16). Given the strong similarities between EFA6 subfamily members in their Sec7-PH-Ct regions (26), we predict that the regulatory modalities found for EFA6A in this study apply to all members of the subfamily. Whether their N-terminal regions, which are more divergent than their Sec7-PH-Ct regions (26) and have functions independent of Arf GTPases (29), add another layer of regulation remains to be investigated.

Table 2. Regulatory regime of Sec7-PH-containing endocytic ArfGEFs

ArfGEF	Arf	Membrane preference	Autoinhibition	Feedback loop	References
Cytohesins	Arf1/Arf6	PIP ₂ /PIP ₃	Yes	Positive	(4, 10, 13, 21)
BRAGs	Arf1/Arf6	Anionic lipids	No	None	(16)
EFA6	Arf1/Arf6	PIP ₂ /PS	No	Negative	(23, 26, 33; this work)

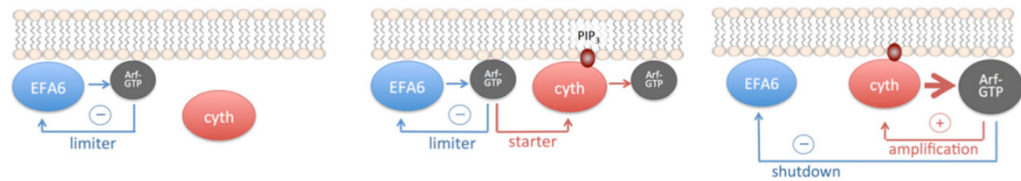


Fig. 4. Model of coupled negative-positive feedback circuit between EFA6 and cytohesins.

Negative and positive feedback loops, such as found in EFA6 and cytohesins, are crucial elements that can be coupled to determine the timing and amplitude of various cellular behaviors (reviewed in ref. 43), suggesting the attractive possibility that EFA6 and cytohesins can leverage their regulatory regimes to work in concert. Remarkably, the negative feedback loop in EFA6 would provide a simple solution to the absolute requirement of cytohesins for activation by Arf-GTP before their GEF activity is amplified by a positive feedback loop (13) (Fig. 4). Being devoid of constitutive autoinhibition by its PH domain or of a strict requirement for a signaling lipid for its recruitment to the plasma membrane, EFA6 could produce basal levels of Arf-GTP, with its negative feedback loop working initially as a limiter (Fig. 4, *Left*). Cytohesins, on recruitment to the plasma membrane induced by an increase in PIP₂ or PIP₃, could then mobilize this dormant Arf-GTP pool to trigger their own GEF activity (Fig. 4, *Center*). The resulting burst in Arf-GTP and Arf1-GTP production would then concurrently sustain the GEF activity of cytohesins and turn off the GEF activity of EFA6, making cytohesins the major active ArfGEFs at that stage (Fig. 4, *Right*). In this model, EFA6 and Arf6 would have a general (although not necessarily unique) function in tuning the level of cytohesin activity.

The foregoing model involving an ArfGEF circuit with differential activation of Arf1 and Arf6 is supported by certain lines of evidence. Notably, a separation of Arf6 activation and effector functions has been observed in endocytic receptor recycling, in which a dormant pool of Arf6-GTP was observed in clathrin-coated pits that became mobilized only later in fast endocytic events (44). This finding would be consistent with the proposed role of EFA6 in sustaining a basal amount of Arf6-GTP. Likewise, it has been shown that Arf1 and Arf6 have different peaks of activation during phagocytosis, with Arf1 activation delayed and lasting longer than Arf6 activation, although the GEFs involved are unknown (38). Recent work also showed that Arf6 and Arf1 must be activated in sequence for WRC-mediated actin assembly, and that this requires a cytohesin and either EFA6 or BRAG working in concert (45).

It should be noted that a corollary of the feedback circuit model is that expression of dominant active or inactive Arf GTPases or of ArfGEFs and ArfGEF mutants is predicted to interfere with multiple, possibly opposing steps, which should be taken into account when interpreting the resulting phenotypes. Accordingly, the challenge to investigate such feedback circuit will be to find ways to perturb and monitor individual steps separately. Future investigations of the structural mechanism whereby Arf6-GTP and the PH-Ct domain of EFA6 establish the negative feedback loop should be an important step toward this goal.

Experimental Procedures

Expression and Purification of Recombinant Proteins. Arf1 and Arf6 constructs were expressed in *Escherichia coli*, purified, and, when appropriate, modified by coexpression or *in vitro* myristoylation and/or loaded with GDP or GTP nucleotides as described previously (16, 34). Cloning,

expression, and purification of human EFA6^{Sec7} (residues 527–727), EFA6^{Sec7-PH-Ct} (residues 527–1024), and EFA6^{PH-Ct} (residues 730–1024) are described in *SI Experimental Procedures*. EFA6^{PH-Ct} and EFA6^{Sec7-PH-Ct} were further characterized by synchrotron SAXS (Fig. S1A) and by size exclusion chromatography with multiangle light scattering (Fig. S1B), respectively, which demonstrated that the constructs are monomers, and thus the C-terminal domain does not form a homodimeric coiled coil.

Cell Culture, Reagents, and Antibodies. Baby hamster kidney cells (BHK-21) were grown in BHK-21 medium (Gibco-BRL), containing 5% FCS, 10% Tryptose phosphate broth, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Mouse mAb against the myc epitope (clone 9E10; Roche Diagnostics) was obtained from Jackson ImmunoResearch.

Liposomes. All lipids were obtained from Avanti Polar Lipids. Liposomes were prepared as described previously (13) in a buffer containing 50 mM Hepes pH 7.4 and 120 mM potassium acetate. Unless specified otherwise, liposomes contained 34% phosphatidylcholine (PC), 14% phosphatidylethanolamine (PE), 21% phosphatidylserine (PS), 0.7% phosphatidylinositol-4,5-diphosphate (PIP₂), and 30% cholesterol and were extruded through a 0.2-μm filter (Whatman).

Nucleotide Exchange Kinetics. Nucleotide exchange kinetics were performed by monitoring tryptophan fluorescence ($\lambda_{\text{exc}} = 298 \text{ nm}$; $\lambda_{\text{exc}} = 340 \text{ nm}$) on a fluorimeter (Cary) equipped with stirring and thermostating devices. Experiments were performed in HKM buffer (50 mM Hepes buffer, pH 7.4, 120 mM potassium acetate, and 1 mM MgCl₂) supplemented with 1 mM DTT at 37 °C. Pseudofirst-order rate constants (k_{obs}) were obtained by fitting the kinetic traces with exponential functions, except for EFA6^{Sec7-PH-Ct} and ^{myr}Arf6, which had more complex kinetics, as was previously observed with other ArfGEFs (16). In this case, k_{obs} values were derived from initial velocities as described in *SI Experimental Procedures*. k_{cat}/K_M values were determined from k_{obs} measured over a range of GEF concentrations, as described previously (16, 46). The liposome concentration was 100–200 μM, at which the protein/lipid ratio is sufficiently high for most of the lipid surface to remain accessible to the proteins (13).

Binding Experiments. Equilibrium dissociation constants (K_d) of IP₃ to EFA6^{PH-Ct} or EFA6^{Sec7-PH-Ct} were determined from the decrease in fluorescence emission at 340 nm by fitting the following equation: $\Delta F_{340\text{nm}} = \Delta F_{\text{max}} \times [L]/(K_d + [L])$, where $[L]$ is the concentration of IP₃. For pull-down experiments, BHK-21 cells were transfected with plasmids encoding C-terminally myc-tagged Arf6Q67L or Arf6T157N, using Jet Pei reagent. After 24 h, cells were lysed in 50 mM Tris pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1% Triton-X100, 2 mM DTT, and a mixture of protease inhibitors (Roche), and then centrifuged at 15,000 × *g* for 10 min at 4 °C. Supernatants were incubated with 2 μM GST constructs in the presence of 0.75% BSA and glutathione Sepharose beads overnight at 4 °C. The beads were then washed, and bound proteins were eluted using SDS sample buffer and separated on SDS/PAGE.

The presence of Arf6 in the eluate was detected by Western blot analysis using the anti-tag antibodies. For cosedimentation experiments, purified Δ13Arf6-GTP (1.5 μM) and EFA6^{PH-Ct} proteins (0.5 μM) were incubated with sucrose-loaded fluorescent liposomes (34.3% PC, 14% PE, 21% PS, 0.7% PIP₂, 30% cholesterol, and 0.2% NBD-PE) extruded on a 0.2-μm filter. Liposomes were sedimented for 20 min at 400,000 × *g*, checked by fluorescence using a FujiLAS 3000 imager equipped with a CCD camera, and analyzed on 15% SDS/PAGE stained with Sypro-orange and by Western blot analysis with the 1D9 anti-Arf monoclonal antibody (Thermo Fisher Scientific).

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Supporting Information

Padovani et al. 10.1073/pnas.1409832111

SI Experimental Procedures

Expression and Purification of Recombinant Proteins. Human EFA6A^{Sec7} (residues 527–727) and EFA6A^{Sec7-PH-Ct} (residues 527–1024) were cloned into a pProEX HTb plasmid with an N-terminal 6-His tag and tobacco etch virus (TEV) protease cleavage site. Both constructs were overexpressed in *Escherichia coli* and purified essentially as described previously for EFA6A^{Sec7-PH-Ct} (1), except that the 6-His tag was removed by TEV protease treatment (1 mg TEV/10 mg proteins, overnight at 4 °C) before the ion-exchange chromatography step.

Different vectors were used for EFA6A^{PH-Ct} (residues 730–1024). For use in nucleotide exchange experiments, EFA6A^{PH-Ct} with an N-terminal 6-His tag was cloned into pET8C (Novagen) and purified by affinity chromatography on a HisTrap FF column, followed by ion-exchange chromatography on a MonoS column (GE Healthcare). For use in binding and structural experiments, the same sequence with an N-terminal 6-His tag and a TEV cleavage site was subcloned by recombination into the Gateway pDEST17 vector (Invitrogen). The recombinant pro-

tein was produced in Rosetta(DE3)pLysS *E. coli* strains and purified by nickel-affinity chromatography followed by cleavage using TEV protease, followed by a second nickel-affinity chromatography to remove the recombinant TEV protease and then a final gel filtration step using a Superdex 200 16/600 (GE Healthcare) equilibrated with buffer (10 mM Hepes pH 7.5 and 500 mM NaCl). For pull-down experiments, EFA6A^{PH-Ct} was fused to GST and purified from bacteria according to the manufacturer's instructions (GE Healthcare). After elution with glutathione, the purified proteins were dialyzed against 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, and 10% glycerol (dialysis buffer), and then stored at –20 °C.

Determination of k_{obs} from Initial Velocities. For EFA6^{Sec7-PH-Ct} and myr¹Arf6, initial velocities v_i ($\Delta F_{340\text{nm}} \cdot \text{s}^{-1}$) were determined and transformed into k_{obs} (s^{-1}) using a $\Delta \epsilon$ value ($\text{mM}^{-1} \cdot \text{cm}^{-1}$) calculated for each independent experiment from the ΔF_{max} and the concentration of myr¹Arf6.

1. Padovani D, Zeghouf M, Traverso JA, Giglione C, Cherfils J (2013) High yield production of myristoylated Arf6 small GTPase by recombinant N-myristoyl transferase. *Small GTPases* 4(1):3–8.

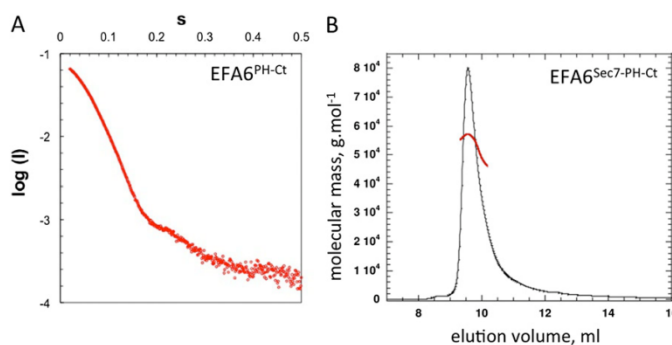


Fig. S1. Characterization of purified recombinant EFA6 constructs. (A) Synchrotron small-angle X-ray scattering (SAXS) analysis of EFA6^{PH-Ct} from which the histidine tag has been cleaved. SAXS data were analyzed with FOXTROT software, yielding a radius of gyration of 24 Å, in agreement with an association of the PH and Ct domains in an essentially globular structure. (B) Size-exclusion profile (monitored by refractometry; dark lines) and the molecular masses (calculated from light-scattering and refractometry data; red lines) of EFA6^{Sec7-PH-Ct}. This analysis yielded a molecular mass of 57.2 ± 0.3 kDa ($R_h = 3.46 \pm 0.25$ nm).

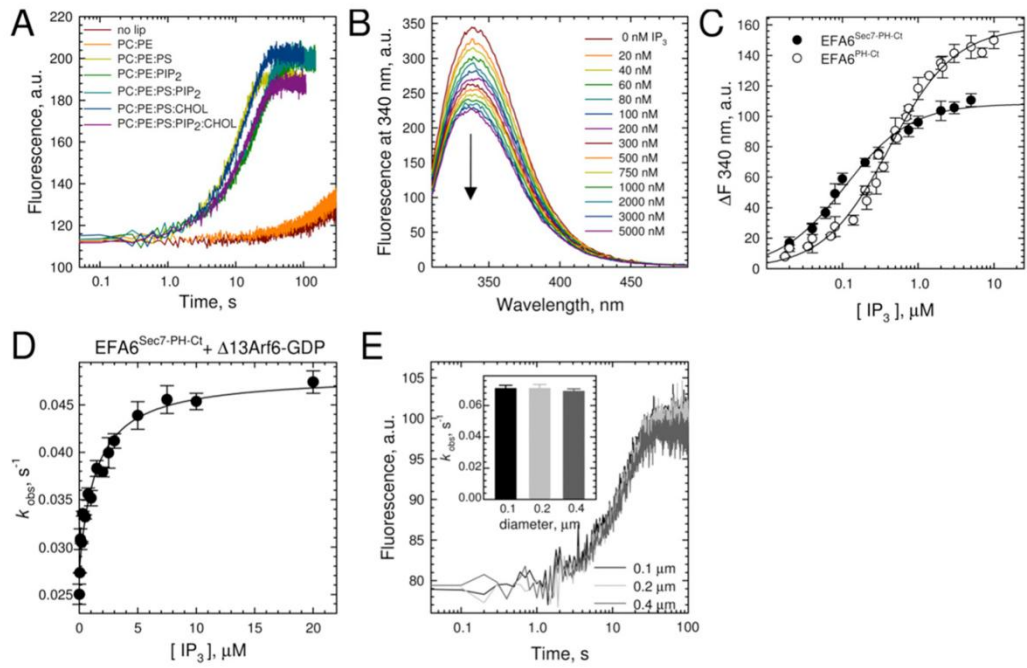


Fig. S2. Sensitivity of EFA6 to membrane composition and curvature. (A) Representative kinetic traces observed for the GDP/GTP exchange of myr Arf6-GDP in the presence of EFA6^{Sec7-PH-Cl} on liposomes of various compositions (given in Table S1). (B) Fluorescence emission spectra ($\lambda_{exc} = 280$ nm) of EFA6^{Sec7-PH-Cl} (1 μ M) recorded in HKM buffer supplemented with 1 mM DTT at 20 °C in the presence of increasing concentrations of IP₃ (up to 5 μ M). The addition of IP₃ induced a quenching of fluorescence as well as a slight red shift. (C) Equilibrium binding constant of IP₃ for EFA6^{Sec7-PH-Cl} and EFA6^{PH-Cl} determined by fluorescence. K_D values are 109 ± 9 nM and 417 ± 24 nM, respectively. (D) Effect of IP₃ on the EFA6^{Sec7-PH-Cl}-catalyzed exchange reaction of Δ 13Arf6-GDP in solution. A hyperbolic fit to the data gave $k_{act}(IP_3) = 1.40 \pm 0.30$ μ M at 37 °C. (E) The EFA6^{Sec7-PH-Cl}-catalyzed exchange reaction of myr Arf6-GDP is not sensitive to membrane curvature. The diameter of liposomes is indicated.

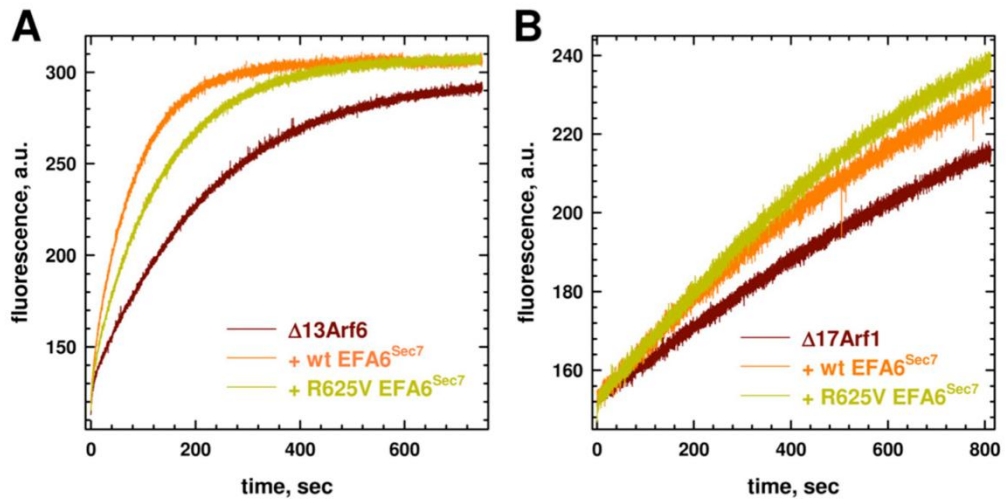


Fig. 53. Effect of the Arg625Val mutation on EFA6^{Sec7} catalytic activity. Representative kinetic traces observed for the GDP/GTP exchange reaction of 1 μ M Δ 13Arf6-GDP (A) or Δ 17Arf1-GDP (B) alone or in the presence of EFA6^{Sec7} or EFA6^{Sec7-R625V} (400 nM in A, 1 μ M in B). k_{obs} values (s^{-1}) for Δ 13Arf6 are 0.0043 for spontaneous, 0.0117 for EFA6^{Sec7}, and 0.0073 for EFA6^{Sec7-R625V}. k_{obs} values for Δ 17Arf1 are 0.0007 for spontaneous, 0.0011 for EFA6^{Sec7}, and 0.0011 for EFA6^{Sec7-R625V}.

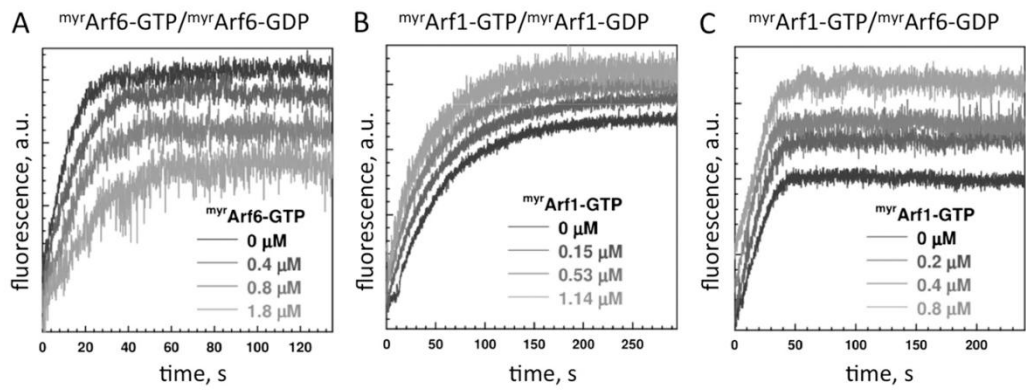


Fig. 54. EFA6 discriminates between Arf1-GTP and Arf6-GTP as effectors of the negative feedback loop. Kinetics experiments were carried out as described in Fig. 2A. The second part of the reaction is aligned as in Fig. 2B. The different ^{myr}Arf-GTP/^{myr}Arf-GDP combinations are indicated.

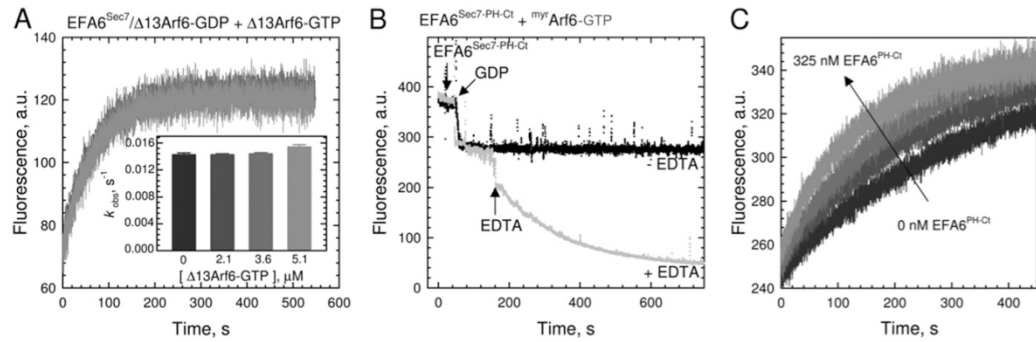


Fig. 55. Mechanism of EFA6 regulation by Arf6-GTP. (A) $\Delta 13\text{Arf6-GTP}$ does not compete with $\Delta 13\text{Arf6-GDP}$. EFA6^{Sec7} (500 nM) exchange activity on $\Delta 13\text{Arf6-GDP}$ (0.7 μM) was monitored in the presence of increasing concentrations of $\Delta 13\text{Arf6-GTP}$ (up to 5.1 μM). The absence of effect indicates that the negative feedback loop does not result from a competition mechanism. (B) myrArf6-GTP is not a substrate of EFA6^{Sec7-PH-Ct}. EFA6^{Sec7-PH-Ct} (20 nM) does not stimulate the exchange of GTP for GDP from myrArf6-GTP (0.4 μM). GTP-GDP exchange in the presence of 1 mM EDTA (no EFA6) is shown for comparison. (C) EFA6^{PH-Ct} reverses the negative feedback effect in a dose-dependent manner. Nucleotide exchange experiments were carried out with EFA6^{Sec7-PH-Ct}, myrArf6-GDP, and liposomes in the presence of increasing concentrations of EFA6^{PH-Ct}.

Table S1. Sensitivity of EFA6^{Sec7-PH-Ct} to liposome composition

Liposome	myrArf6-GDP		myrArf1-GDP	
	$k_{\text{obs}}, \text{s}^{-1}$	Fold increase	$k_{\text{obs}}, \text{s}^{-1}$	Fold increase
None	0.00135 ± 0.00090	1	0.00037 ± 0.00012	1
PC:PE	0.00477 ± 0.00015	3.5	0.00094 ± 0.00006	2.6
PC:PE:PS	0.1275 ± 0.0162	95	0.0960 ± 0.0057	260
PC:PE:PIP ₂	0.0518 ± 0.0041	38	0.0580 ± 0.0015	157
PC:PE:PS:PIP ₂	0.0650 ± 0.0039	48	0.0230 ± 0.0014	62
PC:PE:PS:CHOL	0.0990 ± 0.0087	73	0.0598 ± 0.0027	162
PC:PE:PS:PIP ₂ :CHOL	0.0789 ± 0.0065	59	0.0275 ± 0.0014	75

CHOL, cholesterol.

Experiments were carried out using 0.4 μM myrArf6-GDP and 11 nM EFA6^{Sec7-PH-Ct} or 0.4 μM myrArf1-GDP and 13 nM EFA6^{Sec7-PH-Ct}. All values are the average \pm SD of three independent experiments. The lipid composition of the liposomes is as follows (% total lipids): PC:PE, PC 80, PE 20; PC:PE:PS, PC 50, PE 20, PS 30; PC:PE:PIP₂, PC 78, PE 20, PIP₂ 2; PC:PE:PS:PIP₂, PC 48, PE 20, PS 30, PIP₂ 2; PC:PE:PS:CHOL, PC 35, PE 14, PS 21, CHOL 30; PC:PE:PS:PIP₂:CHOL, PC 34.3, PE 14, PS 21, PIP₂ 0.7, CHOL 30.

D. Conclusion

Dans cette étude menée par l'équipe du Dr Cherfils et à laquelle j'ai pu participer, nous avons pu mettre en évidence que, contrairement au facteur d'échange ARNO, le domaine PH d'EFA6 ne possède pas d'effet inhibiteur sur son activité catalytique. Par ailleurs, nous avons constaté que sa présence potentialisait l'échange nucléotidique d'Arf6 myristylée catalysée par le domaine Sec7 sur des vésicules composées de PIP2 et de PS. De manière intéressante, nous avons également mis en évidence que l'activité catalytique de la protéine EFA6 est inhibée par son produit Arf6-GTP. Arf6-GTP, le produit de la réaction catalysée par EFA6, va réguler négativement l'activité de ce dernier en interagissant avec son domaine PH-C-terminal.

L'ensemble de ces résultats a permis de proposer un modèle selon lequel EFA6 serait régulé par un rétrocontrôle négatif induit par l'interaction de son produit Arf6-GTP avec son domaine PH-C-terminal.

Les boucles de rétrocontrôle positif et négatif, comme celles régulant l'activité d'ARNO et d'EFA6, sont des phénomènes essentiels et nous pouvons imaginer qu'ils peuvent être couplés pour contrôler différentes voies de signalisation au sein des cellules. Dans notre modèle, EFA6 serait capable d'activer un pool d'Arf6 qui viendrait exercer un rétrocontrôle et inhiber son activité. Lorsque la protéine ARNO serait recrutée à la membrane plasmique, ce pool d'Arf6-GTP viendrait lever son auto-inhibition et ainsi permettre l'activation des protéines Arf1 et Arf6. Ce phénomène amplifierait l'effet activateur sur ARNO et l'inhibition sur EFA6 induite par Arf6-GTP. Dans ce modèle EFA6 et Arf6 serviraient à l'activation d'ARNO qui deviendrait le facteur d'échange le plus actif dans ces conditions.

Toutefois il serait intéressant d'approfondir ces résultats et de déterminer les mécanismes moléculaires par lesquels Arf6-GTP et le domaine PH-C-terminal exercent leur effet inhibiteur.

III. Mécanismes de régulation du facteur d'échange EFA6

A. Introduction

Comme nous avons pu le voir dans l'introduction, les récepteurs couplés aux protéines G, suite à leur activation par leur ligand spécifique vont contrôler plusieurs voies de signalisation par l'intermédiaire de leurs protéines G hétérotrimériques. Cependant, afin que cette activation et la cascade de signalisation induite ne perdurent pas dans la cellule, les RCPGs activés vont être internalisés. Une fois internalisés les récepteurs vont être emmenés au niveau de compartiments intracellulaires endosomaux. Ils vont pouvoir être ensuite soit recyclés rapidement vers la membrane plasmique à partir des endosomes précoces, soit plus tardivement à partir des endosomes de recyclage. Sous stimulation prolongée par un agoniste ces récepteurs vont être dirigés vers les lysosomes et la voie de dégradation. La signalisation de ces récepteurs est un processus finement régulé au sein de la cellule. Cette régulation fait tout d'abord intervenir une protéine kinase de la famille des GRK. Les GRKs appartiennent à la famille des Sérine/Thréonine kinases et vont phosphoryler les RCPGs suite à leur activation. Cette phosphorylation va permettre d'augmenter l'affinité de ces récepteurs avec un deuxième acteur clé de leur désensibilisation : les arrestines. Les β -arrestines, qui sont ubiquitaires, ainsi recrutées vont permettre dans un premier temps le découplage entre le récepteur et les protéines G hétérotrimériques. Dans un deuxième temps les β -arrestines vont permettre le couplage des récepteurs à internaliser avec la machinerie d'endocytose. En effet grâce à la libération de leur extrémité C-terminal induite par l'interaction avec les RCPGs activés, elles sont capables d'interagir avec la protéine adaptatrice AP-2 et la clathrine et ainsi permettre le recrutement des RCPGs dans les puits recouverts de clathrine. Une étude menée au laboratoire a également mis en évidence que les β -arrestines, via leur domaine C-terminal, interagissent de manière simultanée avec EFA6 et Arf6-GDP suite à l'activation du récepteur β 2-adrénérique. Cette interaction est dépendante du ligand et facilite l'activation d'Arf6 par EFA6.

Pendant la dernière partie de ma thèse je me suis donc intéressée à la régulation des interactions protéine-protéine dans ce complexe à trois partenaires que sont EFA6, Arf6 et la

β -arrestine afin de mieux comprendre le rôle d'EFA6 et d'Arf6 dans le processus d'internalisation.