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Anterograde Trafficking of Nascent α_{2B}-Adrenergic Receptor: Structural Basis and Roles of Small GTPases

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I. OVERVIEW

Similar to many other G protein-coupled receptors (GPCRs), the functionality of α_{2B}-adrenergic receptor (α_{2B}-AR) is dependent on its proper transport to the cell surface. However, compared with the well-understood endocytic and recycling pathways, the molecular mechanism underlying the anterograde trafficking of newly synthesized α_{2B}-AR from the endoplasmic reticulum (ER) through the Golgi to the plasma membrane remains poorly elucidated. Recent studies have revealed that α_{2B}-AR targeting to the cell surface is a highly regulated process, which is coordinated by many intrinsic determinants and regulatory proteins. This chapter will review the roles of recently identified motifs and the Sar1/ARF and Rab GTPases in α_{2B}-AR exit from intracellular organelles and transport from the ER to the cell surface.

II. INTRODUCTION

As the largest superfamily of cell surface receptors, G protein-coupled receptors (GPCRs) regulate a variety of cellular functions through coupling to heterotrimeric G proteins, which in turn modulate the activity of downstream effectors, including adenylyl cyclases, phospholipases, protein kinases, and ion channels (Hanyaloglu & von Zastrow, 2008; Pierce, Premont, & Lefkowitz, 2002; Rosenbaum, Rasmussen, & Kobilka, 2009). The life of GPCRs begins in the endoplasmic reticulum (ER) where they are synthesized. Once correctly folded and properly assembled, GPCRs are able to pass the ER quality control system and exit from the ER, beginning the journey of intracellular trafficking (Dong, Filipeanu, Duvernay, & Wu, 2007). The nascent receptors then pass many intracellular compartments, including the ER-Golgi intermediate compartment (ERGIC), the Golgi, and the trans-Golgi network (TGN), *en route* to the cell surface, which is the functional destination for most GPCRs. An important feature for the cell surface GPCRs is that they may undergo internalization in response to sustained agonist stimulation during which the receptors are transported from the plasma membrane to endosomes. The internalized receptors in endosomes may be sorted to different destinations, including the recycling pathway for return to the cell surface, the lysosomal compartment for degradation, and the Golgi for retrograde transport. Therefore, the balance of these dynamic intracellular trafficking events dictates the amount of the receptors at the plasma membrane, which in turn controls the magnitude of cellular response to a given extracellular signal. Over the past decades, most studies on the intracellular trafficking of GPCRs have focused on the endocytosis and recycling processes. These studies have not only greatly advanced our knowledge about the mechanisms of GPCR trafficking but also revealed physiological functions for the trafficking in regulating receptor signal propagation and in the pathogenesis of human diseases (Hanyaloglu & von Zastrow, 2008; Marchese, Chen, Kim, & Benovic, 2003; Moore, Milano, & Benovic, 2007; Tan, Brady, Nickols, Wang, & Limbird, 2004; Wu, Benovic, Hildebrandt, & Lanier, 1998; Wu, Krupnick, Benovic, & Lanier, 1997; Xia, Gray, Compton-Toth, & Roth, 2003). In contrast, the molecular

mechanism underlying anterograde transport of nascent GPCRs from the ER through the Golgi apparatus to the cell surface and the role of export traffic in the functional regulation of the receptors have just begun to be elucidated.

The progress achieved over the past few years indicates that, similar to the endocytic and recycling pathways, the ER-to-cell surface movement of GPCRs is a highly regulated, dynamic process, which is orchestrated by structural features of the receptors and many regulatory proteins. First, it has been demonstrated that ER export is a rate-limiting step for the cell surface transport of GPCRs (Petaja-Repo, Hogue, Laperriere, Walker, & Bouvier, 2000). Second, a number of studies have recently identified highly conserved hydrophobic sequences, which are required for GPCR export from the ER (Bermak, Li, Bullock, & Zhou, 2001; Carrel, Hamon, & Darmon, 2006; Robert, Clauser, Petit, & Ventura, 2005; Schulein et al., 1998). These studies suggest that, similar to many other plasma membrane proteins, GPCR exit from the ER may be dictated by specific export motifs. Third, cell surface transport of GPCRs is modulated by direct interactions with multiple regulatory proteins such as the receptor activity modifying proteins (RAMPs), the ER chaperone proteins, and accessory proteins which may behave as chaperones/escort proteins, stabilizing receptor conformation and promoting their delivery to the plasma membrane (Dong et al., 2007). Fourth, dimerization (homo- and hetero-dimerization) may also participate in the regulation of GPCR export to the cell surface, likely through influencing their correct folding or assembly in the ER (Bouvier, 2001; Salahpour, Angers, Mercier, Lagace, Marullo, & Bouvier, 2004; Zhang et al., 2009; Zhou, Filipeanu, Duvernay, & Wu, 2006). Finally, GPCR transport from the ER through the Golgi to the cell surface is mediated through distinct pathways, in which the Ras-like Rab GTPases play a crucial role (Dong & Wu, 2007; Filipeanu, Zhou, Claycomb, & Wu, 2004; Filipeanu, Zhou, Fugetta, & Wu, 2006; Wu, Zhao, & He, 2003).

My laboratory has used adrenergic and angiotensin II receptors as representatives to search for the players that control the cell surface targeting of the receptors by addressing two important questions: Are there conserved structural elements in GPCRs which function as motifs dictating their exit from intracellular compartments? And could the export trafficking of GPCRs be selectively regulated by well-defined transport regulators? Over the past several years, we have identified several highly conserved residues essential for the receptors to exit from the ER and the Golgi apparatus (Dong & Wu, 2006; Duvernay et al., 2009a, 2009b; Duvernay, Zhou, & Wu, 2004; Zhou et al., 2006). We have also demonstrated that small GTPases, specifically the Rab and Sar1/ARF subfamilies, may selectively or differentially modulate the anterograde traffic of GPCRs along the secretory pathway (Dong & Wu, 2007; Dong et al., 2010a, 2010b; Dong, Zhou, Fugetta, Filipeanu, & Wu, 2008; Filipeanu et al., 2004, 2006; Wu et al., 2003; Zhang et al., 2009).

In this chapter, we will review the role of structural determinants and small GTPases, specifically the Sar1/ARF and Rab subfamilies, in the regulation of α_{2B}-AR exit from intracellular compartments and transport from the ER to the cell surface. There are three α_2-AR subtypes, designated as α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR, all of which play an important role in regulating sympathetic nervous system, both peripherally and centrally. All three α_2-ARs have similar structural features: whereas the third intracellular loop (ICL3) is quite large with more than 170 amino acid residues, other loops and the termini are relatively short with less than 25 residues.

III. THE STRUCTURAL BASIS OF α_{2B}-AR EXPORT FROM THE ER AND THE GOLGI

Although all three α_2-ARs have a strong similarity in their structures and functions, they are markedly different in their abilities to move to the cell surface. In particular, α_{2C}-AR transports to the cell surface in a cell type- and temperature-dependent fashion. For example, α_{2C}-AR is able to efficiently move to the plasma membrane in some neuroendocrine cells, such as PC12 and AtT20 cells, in a temperature-independent manner, whereas the majority of α_{2C}-AR is arrested in the intracellular compartments including the ER and the Golgi, unable to transport to the cell surface at 37°C in fibroblasts and vascular smooth muscle cells, and reducing temperature may facilitate the cell surface transport of the intracellularly accumulated receptors (Bailey, Eid, Mitra, Flavahan, & Flavahan, 2004; Daunt, Hurt, Hein, Kallio, Feng, & Kobilka, 1997; Jeyaraj, Chotani, Mitra, Gregg, Flavahan, & Morrison, 2001). Such an effect of lowering temperature on α_{2C}-AR translocation may contribute to Raynaud syndrome which is characterized by enhanced peripheral vasoconstriction during cold exposure or emotional stress and can be ameliorated by using α_2-AR antagonists. Interestingly, it has been demonstrated that the intra-cellular accumulation of α_{2C}-AR may be under the control of multiple arginine residues in the C-terminus and hydrophobic residues in the N-terminus which may function as ER retention motifs trapping the receptor in the ER (Angelotti, Daunt, Shcherbakova, Kobilka, & Hurt, 2010; Ma et al., 2001).

In contrast to α_{2C}-AR, both α_{2A}-AR and α_{2B}-AR are normally expressed at the cell surface and recent studies have demonstrated that their transport from the ER to the cell surface is controlled by multiple highly conserved specific motifs. Specifically, the F436, I443, and L444 residues [$F(x)_6IL$ motif] in the C-terminus and a single L48 residue in the first intracellular loop (ICL1) are required for α_{2B}-AR to exit from the ER (Duvernay et al., 2004, 2009b, 2009b), whereas the Y12/S13 motif located in the N-terminus is crucial for α_{2B}-AR export from the Golgi (Dong & Wu, 2006). In addition, the ICL3 may possess signals for the retention of the receptor in the basolateral subdomain in polarized cells (Brady, Wang, Colbran, Allen, Greengard, & Limbird, 2003; Edwards & Limbird, 1999; Keefer, Kennedy, & Limbird, 1994; Keefer & Limbird, 1993; Prezeau, Richman, Edwards, & Limbird, 1999; Saunders, Keefer, Bonner, & Limbird, 1998; Saunders & Limbird, 2000; Wozniak & Limbird, 1996).

A. The C-terminal $F(x)_6IL$ Motif in α_{2B}-AR Export from the ER

Protein export from the ER is a selective process that may be dictated by short, linear sequences called ER export motifs (Kappeler, Klopfenstein, Foguet, Paccaud, & Hauri, 1997; Nishimura & Balch, 1997; Nishimura et al., 1999; Nishimura, Plutner, Hahn, & Balch, 2002; Nufer et al., 2002; Nufer, Kappeler, Guldbrandsen, & Hauri, 2003; Votsmeier & Gallwitz, 2001; Wendeler, Paccaud, & Hauri, 2007). Of various ER export motifs identified, the diacidic motifs have been found in the cytoplasmic C-termini of several membrane proteins such as vesicular stomatitis virus glycoprotein (VSVG), cystic fibrosis transmembrane conductance regulator, and potassium channels (KAT1, TASK-3, and Kir2.1) (Ma et al., 2001; Nishimura & Balch, 1997; Nishimura et al., 1999; Sevier, Weisz, Davis, & Machamer, 2000; Wang et al., 2004b; Zuzarte, Rinne, Schlichthorl, Schubert, Daut, & Preisig-Muller, 2007) and demonstrated to function as ER export motifs. Interestingly, export function of the diacidic motifs is mediated through their interaction with components of COPII transport vesicles, particularly Sec24 subunits. This interaction results in the concentration of cargo in ER exit sites and facilitates cargo recruitment onto the vesicles (Farhan, Reiterer, Korkhov, Schmid, Freissmuth, & Sitte, 2007).

The C-terminal tails of GPCRs consist of a putative amphipathic 8th α-helix in the membrane-proximal region and a nonstructural membrane-distal region. The function of the C-terminus, particularly the membrane-proximal 8th α-helix portion, in regulating cell surface transport of the receptors has been described for a number of GPCRs including angiotensin II type 1 receptor (AT1R), rhodopsin, vasopressin V2 receptor, dopamine D1 receptor, adenosine A1 receptor, melanin-concentrating hormone receptor 1, and luteinizing hormone/choriogonadotropin receptor (Duvernay et al., 2004; Gaborik, Mihalik, Jayadev, Jagadeesh, Catt, & Hunyady, 1998; Heymann & Subramaniam, 1997; Pankevych, Korkhov, Freissmuth, & Nanoff, 2003; Rodriguez, Xie, Wang, Collison, & Segaloff, 1992; Tetsuka, Saito, Imai, Doi, & Maruyama, 2004). We first demonstrated that deletion of the entire C-terminus almost abolished the cell surface expression of α_{2B}-AR and subsequent mutagenesis of individual residues in the C-terminus revealed F436 and I443/L444 residues in the membrane-proximal portion essential for α_{2B}-AR transport to the cell surface (Duvernay et al., 2004) (Fig. 1A). Consistent with the lack of cell surface expression, the mutated receptor lacking the F436 and I443/L444 was unable to initiate downstream signaling, such as activation of ERK1/2 (Duvernay et al., 2004). Further subcellular distribution analysis showed that the mutated receptors were strongly accumulated in the ER, suggestive of defective ER export. Interestingly, the function of F436 and I443/L444 in mediating α_{2B}-AR export cannot be fully substituted by any other hydrophobic residues (Duvernay et al., 2009b). These data indicate that the $F(x)_6IL$ motif modulates α_{2B}-AR export at the level of the ER and this function is mediated by its unique properties.

Consistent with the role of the $F(x)_6IL$ motif in α_{2B}-AR transport, several similar motifs, such as the $E(x)_3LL$, $FN(x)_2LL(x)_3L$, and $F(x)_3F(x)_3F$ motifs, have been identified to control the ER-to-cell surface transport of other GPCRs (Bermak et al., 2001; Robert et al., 2005; Schulein et al., 1998). Importantly, the $F(x)_6LL$ motif (where x can be any residues and L leucine or isoleucine) is highly conserved in the membrane-proximal C-termini of many family A GPCRs (Duvernay et al., 2004) and indeed, this motif is also required for ER export of several other GPCRs, including α_{1B}-AR, β_2-AR, and AT1R (Duvernay et al., 2009b).

To further provide insights into how the $F(x)_6IL$ motif controls α_{2B}-AR transport, we analyzed the structural features of the motif by homology modeling based on the newly published crystal structure of β_2-AR. F436 residue is buried within the hydrophobic core of the receptor and in close proximity to V42 in the first transmembrane domain and mutation of V42 also significantly impairs α_{2B}-AR export to the cell surface (Fig. 1A). Furthermore, the defect in the transport of the F436A mutant can be partially rescued by a number of treatments, such as chemical chaperones and lowing temperature, and the mutant has enhanced abilities to bind to the chaperone proteins calnexin and calreticulin. These data suggest that the F436 residue is likely involved in the regulation of proper α_{2B}-AR folding in the ER, which is mediated through intramolecular interactions with other hydrophobic residues, such as V42 in the first transmembrane domain, enabling the receptor to pass the ER quality control and to export from the ER.

How I443/L444 residues influence α_{2B}-AR export from the ER remains unknown. The dileucine-based motifs have been demonstrated to be involved in both endocytosis and basolateral delivery. The fact that the branched carbon side chains of the I443/L444 residues are exposed to the cytosolic space suggests that they are capable of providing a docking site for other proteins (Duvernay et al., 2009b). Indeed, our recent studies have demonstrated that Rab8 GTPase modulates β_2-AR transport from the TGN, which is likely mediated through its physical association with the C-terminal dileucine motif of the receptor. However, mutation of the I443/L444 residues did not alter α_{2B}-AR interaction with Rab8 (Dong et al., 2010a). Therefore, to search for proteins interacting with the dileucine motif in

the cytoplasm, particularly components of transport machinery or other trafficking-related regulatory proteins, will help to elucidate the mechanism of the I443/L444 motif in α_{2B}-AR export from the ER.

B. The L48 Residue in α_{2B}-AR Exit from the ER

The ICL1 of α_{2B}-AR is very short, composed of only 12 amino acid residues. Similar to the C-terminus, the ICL1 is absolutely necessary for proper transport of α_{2B}-AR to the cell surface, as the ICL1-deleted receptor was accumulated in intracellular compartments and unable to transport to the cell surface (Duvernay et al., 2009a). Mutagenesis studies identified a single L48 residue essential for the cell surface transport of α_{2B}-AR (Duvernay et al., 2009a) (Fig. 1A) and the mutated receptor was very well co-localized with the ER marker DsRed2-ER (Fig. 2A), suggesting that L48 residue is involved in the regulation of α_{2B}-AR exit from the ER.

An isolated leucine residue in the center of the ICL1 is remarkably conserved among the class A GPCRs. About 85% of the family A GPCRs in human and 83% in all species contain a leucine residue in the center of ICL1 (Duvernay et al., 2009a). Mutation of this conserved residue also significantly attenuated the cell surface expression of several other GPCRs, including β_1-AR, AT1R, and α_{1B}-AR (Duvernay et al., 2009a). These data suggest that the single leucine residue in the ICL1 may be a common signal mediating the ER export of a number of GPCRs.

C. The N-terminal Y12/S13 Motif in α_{2B}-AR Export from the Golgi

Recent studies have demonstrated that, similar to exit from the ER, protein export from the Golgi/TGN is a selective process that may be dictated by specific export motifs. Newly synthesized proteins are sorted at the Golgi/TGN to be delivered to final cellular destinations, such as endosomes, lysosomes, and the plasma membrane. There are several well-defined endosomal sorting signals including tyrosine-based motifs (NPxY and YxxØ, where x can be any residue and Ø is a hydrophobic residue) and dileucine-based motifs ([D/E]xxxL[L/I] and DxxLL). Whereas YxxØ and [D/E]xxxL[L/I] motifs are recognized by the adaptor protein complexes, DxxLL is recognized by Golgi-localized γ-ear-containing ARF1-binding proteins (GGAs) (Hirst, Lui, Bright, Totty, Seaman, & Robinson, 2000; Puertollano, Aguilar, Gorshkova, Crouch, & Bonifacino, 2001; Puertollano, Randazzo, Presley, Hartnell, & Bonifacino, 2001). These motifs function to sort protein transport from the TGN to the endosomal compartment (Boucher, Larkin, Brodeur, Gagnon, Theriault, & Lavoie, 2008; Chen, Yuan, & Lobel, 1997; Hou, Suzuki, Pessin, & Watson, 2006; Johnson & Kornfeld, 1992; Lori, Florencia, & Frederick, 2007).

The fact that G protein-coupled olfactory and chemokine receptors as well as the opsin mutant E150K are released from the ER, but accumulated in the Golgi (Gimelbrant, Haley, & McClintock, 2001; Venkatesan, Petrovic, Van Ryk, Locati, Weissman, & Murphy, 2002; Zhu et al., 2006) suggests that GPCR export from the Golgi and transport from the Golgi to the cell surface is a regulated process. We found that the N-terminus, specifically Y12 and S13 residues in the membrane-proximal N-terminal region, is absolutely required for the transport of α_{2B}-AR to the cell surface. Single and double substitution of the Y12/S13 motif significantly reduced the cell surface expression of α_{2B}-AR (Dong & Wu, 2006) (Fig. 1A). However, unlike the $F(x)_6IL$ and L48 mutants that were accumulated in the ER, the Y12/S13 motif mutants were retained in the Golgi apparatus (Dong & Wu, 2006) (Fig. 2B), suggesting that the Y12/S13 motif mediates α_{2B}-AR export at the level of the Golgi. The YS motif only exists in the membrane proximal N-termini of three α_2-AR family members and indeed, it exerts a similar function on α_{2A}-AR trafficking (Dong & Wu, 2006). Therefore,

the YS motif may function as an export signal specifically modulating the Golgi export of the members of α_2-AR subfamily.

In addition to α_{2B}-AR, an important role for the N-terminus in the intracellular trafficking of GPCRs has been described for other GPCRs. For example, the deletion of the N-termini facilitates the cell surface export of α_{1D}-AR and α_{2C}-AR, suggesting that the N-termini may contain signals retaining the receptors in the ER (Angelotti et al., 2010; Hague, Chen, Pupo, Schulte, Toews, & Minneman, 2004). Taken together, these studies demonstrate that, similar to the C-termini, the N-termini may also contain signals modulating the export of GPCRs from intracellular compartments.

The Y12/S13 motif represents the first Golgi export motif identified in the GPCR superfamily. As the N-terminus is positioned towards the lumen of ER and Golgi during the export process, the YS motif is not able to directly interact with components of transport machinery in the cytoplasm. Furthermore, the fact that YS mutant receptors are able to exit from the ER to reach the Golgi compartment suggests that they are properly folded. Therefore, the defective transport is unlikely caused by misfolding. Further investigation is needed to clarify the molecular mechanism underlying the function of YS motif in the regulation of receptor export from the Golgi.

D. The ICL3 in the Basolateral Targeting of Three α_2-ARs

It has been well demonstrated that the ICL3 is involved in the regulation of receptor coupling to G proteins, phosphorylation, internalization, and signal termination (DeGraff, Gurevich, & Benovic, 2002; Jewell-Motz, Small, Theiss, & Liggett, 2000; Pao & Benovic, 2005; Small, Brown, Forbes, & Liggett, 2001; Wade, Lim, Lan, Chung, Nanamori, & Neubig, 1999; Wade, Scribner, Dalman, Taylor, & Neubig, 1996; Wang & Limbird, 2002; Wang et al., 2004a; Wu et al., 1997, 1998; Wu, Bogatkevich, Mukhin, Benovic, Hildebrandt, & Lanier, 2000). The role of the ICL3 in the localization and trafficking of three α_2-ARs have been extensively studied in polarized Madin–Darby canine kidney II (MDCKII) cells in the laboratory of Dr. Lee Limbird (Brady et al., 2003; Edwards & Limbird, 1999; Keefer et al., 1994; Prezeau et al., 1999; Saunders et al., 1998; Saunders & Limbird, 2000; Wozniak & Limbird, 1996). It has been demonstrated that three newly synthesized α_2-ARs use different pathways to target to the basolateral domain and have distinct retention profiles in MDCKII cells. Consistent with different transport abilities of the three α_2-ARs in some cell types, at steady state, both α_{2A}-AR and α_{2B}-ARs are almost exclusively located at the basolateral surface, while about half of α_{2C}-AR is localized at the basolateral membrane and another half in the intracellular compartments. More interestingly, it appears that α_{2A}-AR and α_{2B}-AR utilize different paths for their basolateral targeting. α_{2B}-AR is first *randomly* transported to both the apical and basolateral surfaces and then selectively retained at the basolateral domain, whereas α_{2A}-AR is directly delivered to the basolateral membrane. Despite the remarkable differences in basolateral targeting, three α_2-ARs exhibit comparable half-life of about 10–12 h at the basolateral domain (Wozniak & Limbird, 1996).

The ICL3 and the C-terminus are not involved in the regulation of direct basolateral delivery of α_{2A}-AR and indeed, the basolateral targeting information for α_{2A}-AR is identified in the membrane-embedded regions (Keefer et al., 1994; Keefer & Limbird, 1993; Saunders et al., 1998). However, removal of the ICL3 significantly facilitates the turnover of the cell surface α_{2A}-AR, shortening its half-life to about 4 h (Edwards & Limbird, 1999). This function of the ICL3 in stabilizing α_{2A}-AR and α_{2B}-AR at the basolateral surface is directly linked to its ability to physically associate with spinophilin (Brady et al., 2003; Richman, Brady, Wang, Hensel, Colbran, & Limbird, 2001). Taken together, these data suggest that the stabilization/

retention of α_{2A}-AR and α_{2B}-AR at specific membrane domains is most likely mediated through ICL3 interactions with other proteins.

IV. THE ROLE OF SMALL GTPASE IN THE EXPORT TRAFFICKING OF α_{2B}-AR

The Ras-like small GTPase superfamily consists of more than 150 members and can be divided into Ras, Rho/Rac/Cdc42, Ran, Sar1/ARF and Rab subfamilies. The small GTPases in the Ras and Rho/Rac/Cdc42 subfamilies have been well documented to function as signaling proteins modulating gene expression, cell division, and cytoskeletal reorganization, the small GTPases in the Rab and Sar1/Arf subfamilies regulate vesicle trafficking, and the Ran GTPases regulate nucleocytoplasmic transport (Takai, Sasaki, & Matozaki, 2001). The roles of the small GTPases in the transport of newly synthesized GPCRs from the ER to the cell surface have been recently studied. Through manipulating the function of endogenous small GTPases by expressing their GDP- and GTP-bound mutants and siRNA targeting to specific GTPases, we and others have recently demonstrated that multiple small GTPases in the Sar1/ARF and Rab subfamilies modulate GPCR cell surface transport *en route* from the ER and the Golgi/TGN.

A. Sar1 in α_{2B}-AR Exit from the ER

The small GTPase Sar1 and the heterodimeric Sec23/24 and Sec13/31 complexes are the components of COPII-coated transport vesicles, which exclusively mediate export of newly synthesized cargo from the ER. It has been well demonstrated that GDP/GTP exchange and GTP hydrolysis by Sar1 GTPase play a crucial role in the formation and budding of COPII-coated vesicles on the ER membrane. Assembly of the COPII coat takes place on the ER membrane at discrete locations called ER exit sites and is initiated by the exchange of GDP for GTP on Sar1 GTPase. GTP activation of Sar1 GTPase recruits the Sec23/24 and Sec13/31 complexes onto the ER membrane forming the COPII-coated vesicles. Hydrolysis of GTP to GDP by Sar1 GTPase results in the dissociation of Sar1 GTPase from the ER membrane and the release of the COPII vesicles (Gurkan, Stagg, Lapointe, & Balch, 2006; Pucadyil & Schmid, 2009).

As a first study to define the role of the ER-derived COPII transport vesicles in GPCR export from the ER, we determined the effect of transient expression of the GTP-restricted mutant Sar1H79G, which presumably blocks the release of the COPII vesicles from the ER membrane, on the cell surface expression and subcellular distribution of α_{2B}-AR (Dong et al., 2008). Expression of Sar1H79G significantly attenuated the cell surface expression of α_{2B}-AR (Fig. 1B) and arrested α_{2B}-AR in ER exit sites (Dong et al., 2008). These data indicate that α_{2B}-AR export from the ER and transport to the cell surface is dependent on the normal function of the small GTPase Sar1. These data also suggest that, similar to many other proteins, α_{2B}-AR exit from the ER is mediated through the Sar1-dependent COPII-coated vesicles. Similar to α_{2B}-AR, the cell surface expression of β_2-AR, AT1R, and human calcium receptor (hCaR) was attenuated by Sar1H79G mutant and siRNA-mediated knockdown of Sar1 (Dong et al., 2008; Zhuang, Chowdhury, Northup, & Ray, 2010), further confirming a general role for Sar1 GTPase in the cell surface transport of the GPCR superfamily.

B. ARF GTPases in α_{2B}-AR Exit from the ER and the Golgi

Of the five ARF GTPases (ARF1, 3, 4, 5, and 6) identified in humans, ARF1 and ARF6 are the best characterized and well understood members. ARF6 primarily engages in the regulation of endocytic trafficking and cytoskeleton remodeling, whereas ARF1 recruits different sets of coat proteins to form distinct transport vesicles that control protein transport

at different intracellular organelles (Palacios, Price, Schweitzer, Collard, & D'Souza-Schorey, 2001; Spang, 2002; Stearns, Willingham, Botstein, & Kahn, 1990). For example, ARF1 recruits coatomers in the formation of COPI vesicles, which mediate protein transport from the Golgi to the ER, from the ERGIC to the Golgi, and intra-Golgi traffic, whereas ARF1-mediated recruitment of adaptor proteins and GGA, leading to the formation of the clathrin-coated vesicles on the TGN controls post-Golgi transport between the TGN, the plasma membrane and the endosomal compartment (Bonifacino, 2004). Based on the sequence homology, it is believed that ARF1 and ARF3 share the same function. In contrast, the function of ARF4 and ARF5 remains largely unknown.

We have recently determined the role of each ARF GTPase in the cell surface targeting of α_{2B}-AR (Dong et al., 2010b). Our studies demonstrated that expression of the GDP-bound, GTP-bound, and guanine nucleotide-deficient mutants of both ARF1 and ARF3 produced a profound inhibitory effect on the cell surface expression of α_{2B}-AR, whereas ARF4, ARF5, and ARF6 mutants produced only moderate or no effect. These data indicate that five human ARF GTPases differentially modulate α_{2B}-AR cell surface transport and that ARF1 and ARF3 are the primary ones regulating α_{2B}-AR export trafficking. Interestingly, we have demonstrated that ARF1 is able to physically associate with α_{2B}-AR as measured by coimmunoprecipitation and GST fusion protein pull-down assay and the interaction domain has been mapped to the C-terminus of the receptor (Dong et al., 2010b). These studies suggest that regulation of α_{2B}-AR transport by ARF1 may be mediated through their direct interaction.

It appears that ARF1 GTPase modulates the cell surface transport of α_{2B}-AR at multiple transport steps as the GDP- and GTP-bound ARF1 mutants arrested the receptors in distinct intracellular compartments (Dong et al., 2010b). Whereas expression of the GDP-bound mutant ARF1T31N arrested α_{2B}-AR in the ER, the GTP-bound mutant ARF1Q71L induced an accumulation of the receptors in the post-ER compartments, including ERGIC, Golgi, and TGN (Dong et al., 2010b). These data indicate that expression of different ARF1 mutants blocks the export of the cargo receptors from different subcellular compartments. Such differential regulation of α_{2B}-AR export by the ARF1 GDP- and GTP mutants could be explained by their effects on the formation of transport vesicles from the different intracellular compartments. Expression of the GDP-bound mutant ARF1T31N would block the formation of COPI vesicles to disrupt the retrograde transport system, which will impair the reuse of components of transport machinery and induces defective anterograde trafficking of newly synthesized cargo. On the contrary, expression of the GTP-bound ARF1Q71L mutant would influence the release of the COPI vesicles from the ERGIC and the Golgi or the clathrin-coated vesicles from the TGN, resulting in the accumulation of α_{2B}-AR in these compartments.

Our studies have demonstrated that ARF1 may play a general role in the anterograde trafficking of the GPCR superfamily. In addition to α_{2B}-AR, we have also measured the effect of the ARF1 mutants on the cell surface transport and subcellular distribution of several other GPCRs including β_2-AR, AT1R, and C-X-C chemokine receptor type 4. Similar to their effects on α_{2B}-AR, expression of the ARF1 mutants markedly inhibited the cell surface expression of all three receptors examined and the GDP- and GTP-bound mutants arrested these receptors in different intracellular compartments (Dong et al., 2010b).

C. Rab GTPases in the ER–Golgi-Cell Surface Transport of α_{2B}-AR

Consisting of more than 60 members in mammals and 11 in yeast, Rab GTPases form the largest subfamily of the Ras-related GTPases and function as traffic "cops" to coordinate almost every step of vesicle-mediated transport, particularly the targeting, tethering, and fusion of the transport vesicles. Each Rab GTPase has a distinct subcellular distribution

pattern that correlates with the compartments between which it coordinates the transport (Takai et al., 2001). There are at least three Rab GTPases, Rab1, Rab2, and Rab6, which coordinate protein transport in the early secretory pathway. Rab1 is localized at the ER and the Golgi, and regulates the anterograde transport of proteins from the ER to the Golgi. Rab2 is localized to the ERGIC that works as the first station sorting cargo into anterograde or retrograde transport pathway and coordinates the early event between the ERGIC and the ER. Rab6 mainly locates in the Golgi and regulates the trafficking from the late to early Golgi cisternae and from the Golgi to the ER. In contrast, Rab8 mediates the vesicle-mediated trafficking from the Golgi/TGN to the plasma membrane.

Most studies on the roles of Rab GTPases in the intracellular trafficking of GPCRs have been focused on the events involved in the internalization (Fan, Lapierre, Goldenring, & Richmond, 2003; Murph, Scaccia, Volpicelli, & Radhakrishna, 2003; Seachrist, Anborgh, & Ferguson, 2000). In contrast, much less is known about the involvement of Rab GTPases in GPCR export to the plasma membrane. As an initial approach to investigate the anterograde transport pathways of GPCRs, we have determined the role of Rab1, Rab2, Rab6, and Rab8 GTPases in the cell surface transport of α_{2B}-AR by transiently expressing dominant-negative mutants and siRNA-mediated depletion of individual Rab GTPases. We found that these Rab GTPases differentially modulate α_{2B}-AR transport to the cell surface. Specifically, inhibition of Rab2 and Rab8 function significantly inhibited α_{2B}-AR transport to the cell surface, whereas inhibition of Rab1 and Rab6 function did not produce any effect (Dong & Wu, 2007; Dong et al., 2010a; Wu et al., 2003). These data demonstrate that the cell surface transport of α_{2B}-AR is dependent on the normal function of Rab2 and Rab8, but independent of Rab1 and Rab6, which have been well documented to function as generic regulators for protein transport between the ER and the Golgi.

As discussed above, the expression of GTP-bound mutant ARF1Q71L induced an extensive accumulation of α_{2B}-AR in the Golgi (Dong et al., 2010b), indicating that α_{2B}-AR actually passes the Golgi stacks *en route* to the cell surface. Therefore, Rab1/Rab6-independent transport of α_{2B}-AR strongly implies that α_{2B}-AR uses a nonconventional pathway to move from the ER to the Golgi. However, how this novel pathway operates remains unknown. Compared with other GPCRs, α_{2B}-AR is one of a few GPCRs that do not contain N-linked glycosylation sites in the N-termini. Glycosylation of the receptors occurs during their transport through the Golgi apparatus, resulting in the formation of mature receptors competent for subsequent transport to the cell surface. Whether posttranslational modifications such as N-linked glycoyslation function as one of the determinants for the selection of transport pathways and whether the N-linked glycosylation dictates the receptors into the Rab1/Rab6-coordinated transport need further investigation. In addition, to further study the function of other Rab GTPases in the ER-to-Golgi transport of α_{2B}-AR may provide important insights into this nonclassic transport pathway.

In contrast to α_{2B}-AR, the cell surface transport of other GPCRs including α_1- AR, β-AR, AT1R, AT2R, and hCaR was attenuated by functional inhibition of Rab1, Rab2, Rab6, and Rab8 (Dong & Wu, 2007; Dong et al., 2010a; Filipeanu et al., 2004, 2006; Li et al., 2010; Wu et al., 2003; Zhang et al., 2009; Zhuang, Adipietro, Datta, Northup, & Ray, 2010). These data demonstrate that Rab1 and Rab6 may selectively modulate the transport of distinct GPCRs. These data also suggest that distinct GPCRs that have common structural features, track to the cell surface and couple to heterotrimeric G proteins may utilize different pathways (i.e., Rab1/Rab6-dependent and Rab1/Rab6-independent) for their movement from the ER to the Golgi.

The function of Rab GTPases in regulating GPCR trafficking may be mediated through their direct interactions with the receptors. For example, Rab4, Rab5, Rab7, and Rab11 bind to

AT1R to modulate its endocytic trafficking (Esseltine, Dale, & Ferguson, 2010; Seachrist et al., 2002). We recently demonstrated that both β_2-AR and α_{2B}-AR are able to associate with Rab8 as revealed by coimmunoprecipitation. Interestingly, these two adrenergic receptors use different motifs to bind Rab8. In contrast to β_2-AR using the LL motif to interact with Rab8, α_{2B}-AR uses multiple sites located in the membrane-proximal (TVFN) and distal (PW and QTGW) C-terminus to interact with Rab8 (Dong et al., 2010a). In particular, the residues N433 and P447 likely play a crucial role in mediating α_{2B}-AR interaction with Rab8 as mutation of either one almost abolished the interaction in GST fusion protein pull down assays. These data suggest that different GPCRs (i.e., α_{2B}-AR and β_2-AR) may provide distinct docking sites for Rab8 GTPase to coordinate their export from the TGN (Dong et al., 2010a).

V. CONCLUSIONS AND PERSPECTIVES

The players involved in the cell surface targeting of GPCRs in general or α_{2B}-AR in particular are just beginning to be revealed. Recent studies have demonstrated that export from the ER and the Golgi of α_{2B}-AR is dictated by specific amino acid residues or motifs scattered throughout the receptor and the transport of α_{2B}-AR from the ER through the Golgi to the cell surface along the secretory pathway is coordinated by multiple GTPases (Fig. 3). However, the mechanism underlying the regulation of α_{2B}-AR export trafficking is still largely unknown. First, although several essential sequences for ER export of α_{2B}-AR or many other GPCRs have been identified (Bermak et al., 2001; Duvernay et al., 2004; Oksche, Dehe, Schulein, Wiesner, & Rosenthal, 1998; Robert et al., 2005; Rodriguez et al., 1992; Schulein et al., 1998; Tai, Chuang, Bode, Wolfrum, & Sung, 1999), none of them have been shown to directly interact with components of COPII vesicles. The most interesting experiment probably is to continue to search for such motifs that are able to directly interact with the components of COPII transport vesicles and facilitate α_{2B}-AR recruitment onto the vesicles. Second, the experiment to use different protein–protein interaction strategies to look for proteins interacting with the well-defined export motifs as discussed above will help to elucidate the possible molecular mechanism for these motifs. Third, as it is clear that α_{2B}-AR uses a nonclassic pathway to move from the ER to the Golgi, the immediate experiments are to fully characterize this pathway.

Cell surface targeting of GPCRs is one of the important factors determining the functionality of the receptors. Indeed, dysfunction of GPCRs caused by defective cell surface trafficking is clearly associated with the development of a number of human diseases such as nephrogenic diabetes insipidus, retinitis pigmentosa, and male pseudohermaphroditism. Therefore, to thoroughly understand the mechanism underlying export trafficking of GPCRs will provide a foundation for the development of therapeutic strategies targeting on specific components of the transport pathway.

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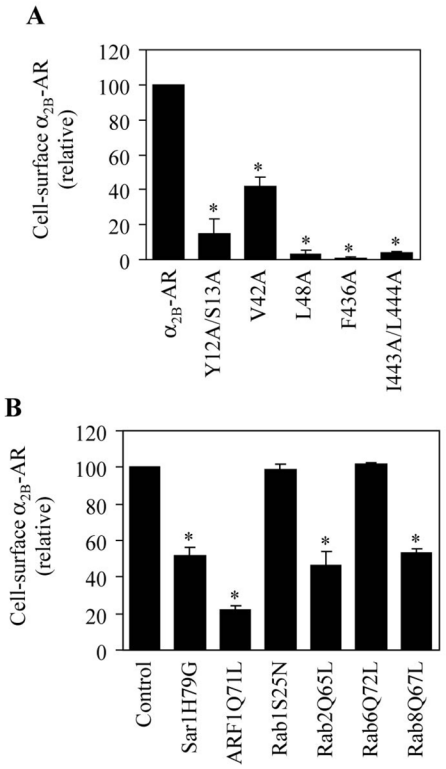

FIGURE 1.

Effect of mutating specific residues (A) and expressing small GTPase mutants (B) on the cell surface expression of α_{2B}-AR as measured by intact cell ligand binding. (A) α_{2B}-AR and its mutants in which specific residues were mutated to alanines were transiently transfected into HEK293 cells. (B) α_{2B}-AR was transfected with or without individual small GTPase mutants into HEK293 cells. The cell surface expression of α_{2B}-AR was measured by intact cell ligand binding by using [^{3}H]-RX821002 at a concentration of 20 nM. *, $p < 0.05$ versus wild type α_{2B}-AR (A) or control (B). (The data are adapted from the references Dong & Wu, 2006; Duvernay et al., 2009a, 2009b).

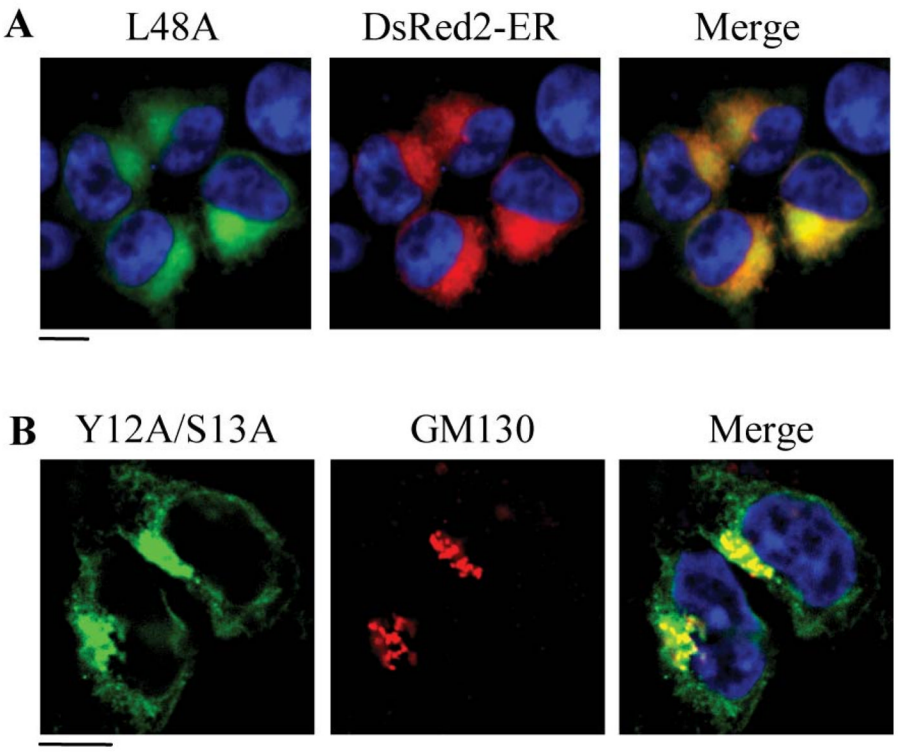

FIGURE 2.

Colocalization of the α_{2B}-AR mutants L48A and Y12A/S13A with ER and Golgi markers, respectively. (A) Colocalization of the α_{2B}-AR mutant L48A with the ER marker DsRed2-ER. HEK293 cells were transfected with the GFP-tagged L48A mutant together with pDsRed2-ER and the subcellular distribution and colocalization of the receptor with DsRed2-ER were revealed by fluorescence microscopy. (B) Colocalization of the α_{2B}-AR Y12A/S13A mutant with the *cis*-Golgi marker GM130. HEK293 cells were transfected with Y12A/S13A and its co-localization with GM130 was revealed by fluorescence microscopy following staining with antibodies against GM130 at 1:50 dilution. Scale bars, 10 μm. (The data are adapted from the references Dong & Wu, 2006; Duvernay et al., 2009a).

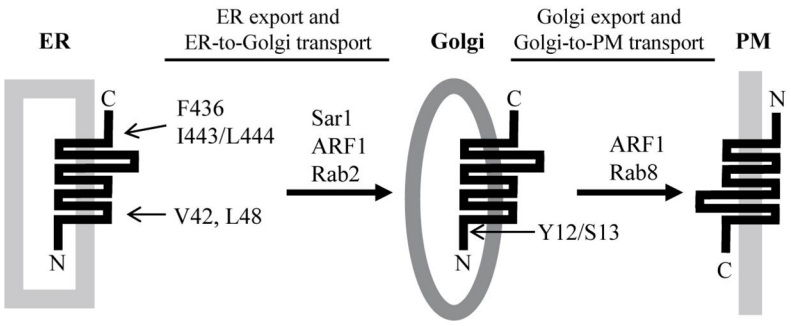

FIGURE 3.

Summary of the structural basis and the roles of small GTPases in the anterograde trafficking of α_{2B}-AR. The F436, I443/L444, V42, and L48 residues regulate the exit of α_{2B}-AR from the ER and the Y12/S13 residues influence the exit from the Golgi. The small GTPase Sar1 controls α_{2B}-AR export from the ER by modulating the function of the COPII vesicles, whereas ARF1 may be involved in the export of α_{2B}-AR from multiple intracellular compartments including the ER and the Golgi. α_{2B}-AR transport from the ER to the Golgi depends on the normal function of Rab2, but independent of Rab1 and Rab6, and its transport from the Golgi to the cell surface requires Rab8.