Published in final edited form as:

Methods Enzymol. 2013 ; 521: 189–202. doi:10.1016/B978-0-12-391862-8.00010-7.

Identification of Endoplasmic Reticulum Export Motifs for G Protein-Coupled Receptors

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Abstract

Coat protein complex II (COPII) vesicle-mediated protein export from the endoplasmic reticulum (ER) can be controlled by linear, independent motifs embedded within the cargo. ER export motifs directly interact with selective components of COPII vesicles and enhance cargo recruitment onto COPII vesicles. Moreover, ER export motifs are able to confer their transport abilities to other proteins. We have recently identified a novel ER export motif for α_{2B} -adrenergic receptor (α_{2B} -AR). This motif selectively interacts with Sec24C/D isoforms of COPII vesicles and facilitates α_{2B} -AR export from the ER as well as transport to the cell surface. This motif can also mediate CD8 glycoprotein transport. These studies indicate that ER export of nascent G protein-coupled receptors (GPCRs) may be directed by specific codes that mediate receptor interaction with the ER-derived COPII vesicles. In this chapter, I discuss experimental approaches to identify ER export motifs for GPCRs by using α_{2B} -AR as a model.

1. INTRODUCTION

Similar to many other plasma membrane proteins, the life of GPCRs begins at the endoplasmic reticulum (ER), where they are synthesized, folded, and assembled. Correctly folded and properly assembled receptors are able to pass through the ER quality control mechanism and to exit the ER. It is well known that exit of nascent cargo from the ER is mediated through coat protein complex II (COPII) transport vesicles that can be formed from three components: the small GTPase Sar1, the Sec23/24 heterodimer, and the Sec13/31 heterodimer (Barlowe et al., 1994; Bickford, Mossessova, & Goldberg, 2004; Gurkan, Stagg, Lapointe, & Balch, 2006; Kuge et al., 1994). A number of recent studies have also demonstrated that the recruitment of cargo onto COPII vesicles is a selective process and can be mediated by ER export motifs.

Several ER export motifs have been identified with diacidic and dihydrophobic motifs being well characterized. The diacidic motifs have been found in the cytoplasmic C-termini of a number of membrane proteins, including vesicular stomatitis viral glycoprotein, cystic fibrosis transmembrane conductance regulator, ion channels, the yeast membrane proteins Sys1p and Gap1p, and G protein-coupled angiotensin II receptors (Ma et al., 2001; Mikosch, Kaberich, & Homann, 2009; Nishimura & Balch, 1997; Nishimura et al., 1999; Votsmeier & Gallwitz, 2001; Wang et al., 2004; Zhang, Dong, Wu, Balch, & Wu, 2011; Zuzarte et al., 2007). The DxE motif (Nishimura & Balch, 1997; Nishimura et al., 1999; Zuzarte et al., 2007), together with neighboring residues (Sevier, Weisz, Davis, & Machamer, 2000), directs the concentration of the cargo molecule during export from the ER, thereby

enhancing the rate of its exit from the ER. The dihydrophobic motifs are required for efficient transport of the ERGIC-53, p24 family of proteins, and the Erv41–Erv46 complex from the ER to the Golgi (Dominguez et al., 1998; Fiedler, Veit, Stamnes, & Rothman, 1996; Nufer, Kappeler, Guldbrandsen, & Hauri, 2003; Otte & Barlowe, 2002).

The function of ER export motifs to enhance cargo transport is mediated through direct interaction with components of COPII vesicles, particularly the Sec24 subunit (Farhan et al., 2007; Mancias & Goldberg, 2008; Miller et al., 2003; Mossessova, Bickford, & Goldberg, 2003; Votsmeier & Gallwitz, 2001; Wang et al., 2004). There are four Sec24 isoforms (Sec24A, Sec24B, Sec24C, and Sec24D) identified in human cells, and based on the sequence homology, they can be further divided into Sec24A/B and Sec24C/D subclasses. Three cargo recognition sites have been mapped in Sec24 (Miller et al., 2003; Mossessova et al., 2003), demonstrating that different ER export motifs interact with the same COPII component to facilitate ER export. Meanwhile, different ER export motifs may have selectivity toward Sec24 isoforms (Mancias & Goldberg, 2008). Based on these studies, ER export motifs should have the following characteristics: (1) modulation of cargo transport at the level of the ER, (2) linear sequence, (3) direct interaction with components of the COPII vesicles, (4) transferable transport properties.

Recent studies from our and other laboratories have identified several motifs essential for GPCR export from the ER and transport from the ER to the cell surface (Bermak, Li, Bullock, & Zhou, 2001; Dong, Filipeanu, Duvernay, & Wu, 2007; Dong & Wu, 2006; Donnellan, Kimbembe, Reid, & Kinsella, 2011; Duvernay, Dong, Zhang, Robitaille, et al., 2009; Duvernay, Dong, Zhang, Zhou, et al., 2009; Duvernay, Zhou, & Wu, 2004; Robert, Clauser, Petit, & Ventura, 2005). However, the molecular mechanisms underlying the function of these motifs in controlling GPCR export remain elusive (Duvernay, Dong, Zhang, Zhou, et al., 2009; Margeta-Mitrovic, Jan, & Jan, 2000; Robert et al., 2005). By using progressive deletion, site-directed mutagenesis, and protein–protein interaction assays, we have recently revealed that a three Arg (3R) motif in the third intracellular loop (ICL3) mediates α_{2B} -adrenergic receptor (α_{2B} -AR) interaction with Sec24C/D isoforms. Mutation of the 3R motif attenuates α_{2B} -AR exit from the ER and transport to the cell surface. Interestingly, the 3R motif can facilitate α_{2B} -AR export trafficking when transferred to the C-terminus. Furthermore, the 3R motif can also enhance CD8 glycoprotein transport to the cell surface (Dong et al., 2012). These data demonstrate that the 3R motif represents a novel ER export motif. In this chapter, I discuss experimental approaches to identify specific sequences/motifs involved in the regulation of ER export of newly synthesized GPCRs.

2. EXPERIMENTAL APPROACHES TO IDENTIFY ER EXPORT MOTIFS FOR GPCRs

2.1. Manipulation of GPCRs

In order to identify the motifs essential for export from the ER, the first step is to select a domain to be manipulated in a given GPCR. The C-terminus and three intracellular loops are positioned toward the cytoplasm which may provide docking sites for components of the ER-derived COPII transport vesicles. In contrast, the N-terminus and three extracellular loops are located in the lumen of the ER and are spatially impossible to directly interact with the transport machinery in the cytoplasm. Therefore, the C-terminus and the intracellular loops should be considered in searching for ER export motifs that are able to physically interact with cytosolic transport machinery.

Once a specific domain is selected to be studied, the next step is to generate the receptor mutant in which the domain of interest is removed and the transport properties of the truncated receptor mutant are compared with those of its wild-type counterpart.

If the deleted mutant indeed is defective in the transport, the deleted domain may contain signals important for receptor transport. If the domain is not too long, such as the C-terminus of α_{2B} -AR which contains only 20 amino acid residues, site-directed mutagenesis can be directly applied to define specific residues that modulate receptor transport. Each of the residues in the domain of interest is mutated to alanine and then the effect of mutations on receptor transport is determined. If site-directed mutagenesis is cumbersome for a domain which is relatively large, the progressive deletion strategy can be utilized to search for a subdomain containing the export signal.

2.2. Measurement of receptor expression at the cell surface

If the cell surface expression of a receptor mutant, in which the domain of interest is deleted or individual amino acid residue mutated, is significantly lower than that of its wild-type counterpart, the deleted domain or the mutated residue is important for receptor export to the cell surface. The cell surface expression of GPCRs can be quantitated by a number of well-established methods, such as ligand binding, flow cytometry, and biotinylation. Our laboratory has established a ligand binding assay in intact living cells for quantifying the cell surface numbers of adrenergic and angiotensin II receptors (Dong & Wu, 2007; Dong, Zhou, Fugetta, Filipeanu, & Wu, 2008; Duvernay, Dong, Zhang, Robitaille, et al., 2009; Duvernay, Dong, Zhang, Zhou, et al., 2009; Filipeanu, Zhou, Fugetta, & Wu, 2006). This method has been used to study the role of Ras-like small GTPases and specific motifs in modulating the intracellular trafficking of GPCRs (Dong & Wu, 2006; Duvernay, Dong, Zhang, Robitaille, et al., 2009; Duvernay, Dong, Zhang, Zhou, et al., 2009; Wang & Wu, 2012). The measurement of the cell surface expression of α_1 -AR, β -AR and angiotensin II receptor by intact cell ligand binding is described elsewhere (Dong & Wu, 2007; Filipeanu, Zhou, Claycomb, & Wu, 2004; Filipeanu et al., 2006; Zhang et al., 2011). The following describes the intact cell ligand binding assay to measure the cell surface expression of α_{2B} -AR using the radioligand [3 H]-RX821002.

HEK293 cells are cultured on 6-well dishes in DMEM and transfected with 1.0 μ g of wild-type and mutated α_{2B} -AR plasmids. After 6 h, the cells are split into 12-well dishes precoated with poly-L-lysine. After 24–36 h transfection, the cells are incubated with DMEM plus [3 H]-RX821002 (PerkinElmer) at a concentration of 20 nM in a total of 400 μ l for 90 min at room temperature. For the nonspecific binding, the cells are incubated with [3 H]-RX821002 plus rauwolscine (Sigma-Aldrich) at a concentration of 10 μ M. The cells are washed twice with 1 ml ice-cold DMEM to remove the excess radioligand. The remained ligands are extracted by digesting the cells with 1 M NaOH for 2 h at room temperature. The liquid phase is collected and 5 ml of Ecoscint A scintillation fluid (National Diagnostics Inc., Atlanta, GA) added. The amount of radioactivity retained is measured by liquid scintillation spectrometry.

Ligand binding of membrane preparations has been long used to quantify GPCR expression. The major advantage of intact cell ligand binding is that it has the ability to accurately measure the numbers of receptors at the plasma membrane, compared with ligand binding of total membrane preparations which contain the receptors expressed in the intracellular compartments, such as the ER and the Golgi. This is particularly important when the mutation or deletion disrupts receptor export, resulting in intracellular retention of the receptor, but does not alter receptor–ligand binding properties.

As mutation of the receptors may alter receptor–ligand binding affinity, other alternative methods such as flow cytometry should be used to confirm the intact cell ligand binding results. For this purpose, the receptors are tagged with an epitope such as FLAG or HA at their N-termini and their expression at the cell surface is measured by flow cytometry following staining with epitope antibodies in nonpermeabilized cells.

In addition to the cell surface expression, it is very important to determine if the mutation or deletion influences the overall synthesis of the receptor, which can be accomplished by a number of methods. If the receptor is tagged with fluorescence proteins such as GFP, the simplest way to measure the overall receptor expression is to directly measure the total fluorescent signal of GFP by flow cytometry or the total GFP expression by immunoblotting using GFP antibodies (Duvernay, Dong, Zhang, Robitaille, et al., 2009).

It is also necessary to exclude the possibility that the reduction in the cell surface expression of a mutated receptor is caused by constitutive receptor internalization. For this goal, receptor internalization blockers can be used to inhibit endocytic transport. If the cell surface expression of a mutated receptor is restored upon coexpression with dominant-negative internalization mutants or treatments with pharmacological blockers, defective export of the mutated receptor is likely caused by facilitated endocytic transport (Dong & Wu, 2006).

In addition to measuring the cell surface expression of the receptor, it is also necessary to define if the mutation of the traffic motif could attenuate signaling elicited by that particular receptor. Although it is expected that the effects of the mutation on the cell surface expression and signaling will be parallel, dysfunction of the mutated receptor will not only confirm the defect in the anterograde transport but also indicate the importance of export process in regulating receptor signal propagation in cells. The selection of functional readouts depends on the type of receptor studied. In our studies, the agonist-mediated activation of the mitogen-activated protein kinases ERK1/2 has been used as a functional readout for several family A GPCRs (Dong & Wu, 2006; Duvernay, Dong, Zhang, Robitaille, et al., 2009; Duvernay, Dong, Zhang, Zhou, et al., 2009).

2.3. Analysis of receptor export from the ER

Once a domain or a specific motif is identified to be important for receptor export, the next step is to directly visualize the subcellular distribution of the mutated receptor by microscopy. If a mutated receptor is extensively localized inside the cell but not on the cell surface, this, together with the reduced cell surface expression measured by intact cell ligand binding, will further indicate that the mutated residue is essential for receptor transport to the cell surface. Microscopic analysis of receptor localization is also able to precisely define the intracellular compartment where the mutated residue modulates receptor export. This could be accomplished by colocalization of the mutated receptors with various intracellular organelle markers (Dong & Wu, 2006; Duvernay, Dong, Zhang, Robitaille, et al., 2009; Duvernay, Dong, Zhang, Zhou, et al., 2009; Duvernay et al., 2004). Analysis of the subcellular colocalization of GFP-tagged α_{2B} -AR with ER markers in fixed cells is described below.

HEK293 cells cultured on 6-well plates are transfected with 100 ng of wild type or mutated α_{2B} -AR tagged with GFP at its C-terminus. Six hours later, the cells are split onto cover slips precoated with poly-L-lysine in 6-well plates. Twenty-four hours following transfection, the cells are washed with ice-cold phosphate buffer saline (PBS) twice with gentle shaking. The cells are fixed with fresh 2 ml of 4% paraformaldehyde–4% sucrose mixture in PBS for 15 min. After washing the cells with 2 ml of ice-cold PBS for 3×5 min, the cells are permeabilized with 2 ml of PBS containing 0.2% Triton X-100 for 5 min. The cells are incubated with 0.24% normal donkey serum (Jackson Immuno) in PBS for 1 h and then with primary antibodies against the ER marker calregulin or calnexin (Santa Cruz) at 1:50 dilution on parafilm with the cover slips upside down for 2 h. Antibodies against other intracellular markers should be used as controls, such as ERGIC53 (Alexis Biochemicals)—an ERGIC marker; GM130 (BD Biosciences)—a *cis*-Golgi marker; p230 (BD Biosciences)—a TGN marker. The cells are washed with cold PBS and incubated with 1 ml of Alexa Fluor 594-labeled secondary antibody (Invitrogen) at a dilution of 1:2000 for 1 h. Following washing

as above, the cells are incubated with 4,6-diamidino-2-phenylindole for 5 min to stain the nuclei. The cover slips are mounted with the mounting media and the fluorescent signal is detected by using an appropriate microscope.

Analysis of the cell surface expression and subcellular distribution of receptors as described above will provide important information about receptor anterograde transport from the ER to the cell surface at the steady status. As receptor transport passes through a number of intracellular compartments such as the ERGIC, Golgi, and TGN en route from the ER to the cell surface, the influences of a specific motif on the cell surface expression and export from the ER may not be identical. It is possible that mutation of an ER export motif significantly slows down the rate of receptor export from the ER, without dramatically reducing receptor cell surface expression and disrupting subcellular localization at the steady status. Therefore, after identifying a motif that modulates receptor export from the ER as discussed above, one should consider measuring the effect of mutating the motif on the kinetics of receptor export from the ER.

The kinetics of receptor export from the ER can be analyzed by fluorescence recovery after photobleaching (FRAP) experiments or by metabolic pulse-chase labeling experiments measuring the formation of complex glycosylated receptors (Bermak et al., 2001; Petaja-Repo, Hogue, Laperriere, Walker, & Bouvier, 2000). The following describes a FRAP protocol that was used in our laboratory to determine the role of the 3R motif in the ER export of α_{2B} -AR in COS7 cells (Dong et al., 2012). As α_{2B} -AR is not accumulated in the Golgi under the normal condition, we took advantage of a previously characterized Golgi-localized YS mutant of α_{2B} -AR (Dong & Wu, 2006) and determined if mutation of the 3R motif could influence the transport of the YS mutant to the Golgi.

COS7 cells are grown on 29-mm glass-bottom culture dishes (In Vitro Scientific) and transfected with either YS mutant in which the YS motif is mutated to Ala or the YS/3R mutant in which both the YS and the 3R motifs are mutated to Ala. Both mutants are tagged with GFP at their C-termini. Prior to imaging, standard media are replaced with CO₂-independent media. FRAP experiments are performed on a Leica SP2-TCS confocal microscope using a 63 $\times$ water immersion objective and the FRAP Wizard application. During imaging, culture dishes are maintained at 37 ° C using a Harvard Apparatus (Holliston, MA) temperature controller and PDMI-2 Micro Incubator. Scan settings are 256 $\times$ 256 pixel resolution at 1400 Hz. For FRAP, 20 prebleach frames are acquired (0.287 s acquisition time/frame), followed by 20 bleach frames (0.287 s/frame) with the 488 laser, and then by 200 post-bleach frames acquired at 5.0-s intervals. The region of interest for bleach/recovery measurement is manually drawn around the Golgi of each cell, and the mean pixel value determined for the region of interest at each recovery time point.

By using this protocol, we found that after photobleaching the intensities of both mutants were markedly reduced and unable to recover to the prebleach intensities, suggesting a large number of the immobilized α_{2B} -AR in the Golgi. The recovery of the YS mutant after photobleaching was much faster than the YS/3R mutant, and the half-time of the recovery of the YS mutant was much shorter than the YS/3R mutant (Dong et al., 2012). These data demonstrate that the mutation of the 3R motif in α_{2B} -AR induces defective transport from the ER to the Golgi.

2.4. Assays for receptor interaction with components of COPII-coated vesicles

Once a motif is identified to modulate receptor export from the ER, the next step is to delineate the possible molecular mechanisms. Because a number of factors, such as proper folding, interactions with accessory proteins, and dimerization, potentially modulate the ER export of GPCRs, disruption of each of these processes could lead to defective export from

the ER. For example, misfolding is the most common cause for the loss of ability of GPCRs to transport from the ER to the cell surface. Indeed, a number of loss-of-function GPCRs, which are associated with many hereditary diseases, are induced by their misfolding, resulting in intracellular accumulation of the receptors. Circular dichroism, treatments with chemical and pharmacological chaperones, and low temperature culture have been used to determine if a GPCR mutant is correctly folded. However, these methods may not work for all kinds of misfolding. Therefore, the folding problem has been very difficult to be excluded in studying GPCR export from the ER.

One important function of ER export motifs is to mediate cargo interaction with components of COPII vesicles. Therefore, it is crucial to determine if the motif is able to interact with the COPII vesicles. We have used glutathione *S*-transferase (GST) fusion protein pull-down, coimmunoprecipitation, and peptide-conjugated agarose affinity matrix to measure the interaction of the 3R motif with four different Sec24 isoforms. GST fusion protein pull-down and coimmunoprecipitation assays have been essentially described in chapter 6 in volume 522 of this series. The following section briefly describes the methods to measure the 3R motif interaction with Sec24 isoforms using the ICL3 peptide-conjugated agarose as affinity matrix (Dong et al., 2012).

The α_{2B} -AR ICL3 peptide NH₂-GKNVGVASGQWRRRTQLSRE-COOH and its mutant NH₂-GKNVGVASGQWWAAATQLSRE-COOH were synthesized, purified by HPLC to >75% and directly conjugated to agarose beads by Biosynthesis Inc. (Lewisville, TX). The peptide-conjugated agarose beads (10 μ l, approximately 13 μ mol peptides) were incubated with 300 μ g cell lysates prepared from HEK293 cells transfected with GFP-tagged Sec24 in 300 μ l of binding buffer containing 20 mM Tris-HCl, pH 7.4, 2% NP-40, 120 mM NaCl at 4 ° C overnight. The resin was washed for four times each with 1 ml of binding buffer. The bound GFP-Sec24 were solubilized in 1 $\times$ SDS gel loading buffer, separated by SDS-PAGE, and detected by immunoblotting using GFP antibodies.

We have demonstrated in both GST fusion protein pull-down and peptide-conjugated affinity matrix that the ICL3 interacted with Sec24C and Sec24D more strongly than with Sec24A and Sec24B (Fig. 10.1A and B) and mutation of the 3R motif markedly reduced their interaction with Sec24 (Fig. 10.1B) (Dong et al., 2012).

2.5. Analysis of the ability of ER export motifs to transport other proteins

Another important property of ER export motifs is to confer their transport abilities to other proteins. For this propose, CD8 glycoprotein has been used in many studies. The following section describes a protocol used in our laboratory to define if the 3R motif could facilitate the transport of CD8 glycoprotein (Dong et al., 2012). To generate the α_{2B} -AR and CD8 chimeras (CD8-WT), the extracellular domains of human CD8 α glycoprotein were generated by PCR using primers (forward primer, 5'-GTCACCTCGAGACCATGGCCTTACCAGTGACCGCC-3' and reverse primer 5'-GTTAGAATTCCCCCGCCGCTGGCCGGCAC-3') and the α_{2B} -AR fragment containing the seventh TM, the C-terminus (from residues 404 to 453) and the ICL3 peptide WWWRRRTQ generated by using primers (forward primer, 5'-GTCAGAATTCTGCAAGGTACCGCATGGC-3' and reverse primer, 5'-GTAC AAGCTT TCA CTG TGT CCG TCT GCG CC-3'). The two PCR products were digested with *Xho*I/*Eco*RI and *Eco*RI/*Hind*III, respectively, and then ligated into the pcDNA3.1 (–) vector (Fig. 10.2A). To measure cell surface expression of α_{2B} -AR-CD8, HEK293 cells were transfected with α_{2B} -AR-CD8 chimeric proteins. The cells were incubated with FITC-conjugated human CD8 antibodies (eBioscience) at 4 ° C for 30 min. To measure the total expression level of α_{2B} -AR-CD8, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice before staining with anti-CD8 antibodies.

We found that the cell surface expression of the chimera CD8-3A was markedly lower than that of the chimera CD8-3R (Fig. 10.2B). Consistently, immunostaining studies revealed cell surface expression of the chimeric protein CD8-3R, whereas CD8-3A was almost undetectable at the cell surface (Dong et al., 2012). These data demonstrate that the 3R motif in α_{2B} -AR is sufficient to direct the export of CD8 glycoprotein to the cell surface.

3. CONCLUSIONS

It is becoming increasingly appreciated that membrane proteins may use specific codes to exit from intracellular compartments, particularly the ER. A number of motifs have also been identified to be required for GPCR export from the ER. However, the molecular mechanisms remain poorly understood. Therefore, continuing search for linear, independent ER export motifs that not only mediate GPCR interaction with components of the COPII vesicles but also confer the transport ability to facilitate the ER exit of other proteins will provide important insights into how newly synthesized GPCRs exit from the ER compartment.

Acknowledgments

This work was supported by National Institutes of Health Grant R01GM076167.

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Figure 10.1.

Interaction of the third intracellular loop of α_{2B} -AR with different Sec24 isoforms. (A) The interaction of the third intracellular loop (ICL3) of α_{2B} -AR with Sec24A, Sec24B, Sec24C, and Sec24D isoforms as determined in GST fusion protein pull-down assay. The ICL3 of α_{2B} -AR was generated as GST fusion proteins. Sec24 isoforms tagged with GFP were transiently expressed in HEK293 cells and total cell homogenates were incubated with GST or GST-ICL3 fusion proteins. (B) The interaction of the ICL3 with Sec24 isoforms as measured by ICL3 peptide-conjugated agarose matrix. The ICL3 fragment G349-E369 containing the 3R motif (3R) or its mutant in which the 3R motif was mutated to three alanines (3A) was synthesized and conjugated to agarose. The ICL3-conjugated agarose beads were incubated with total cell homogenates containing GFP-tagged Sec24 isoforms. In both (A) and (B), bound Sec24 isoforms were revealed by immunoblotting using GFP antibodies (Dong et al., 2012).

Figure 10.2.

Effect of the 3R motif of α_{2B} -AR on the cell surface export of CD8 glycoprotein. (A) A diagram showing generation of chimeric α_{2B} -AR-CD8 proteins. The truncated α_{2B} -AR containing the seventh transmembrane (TM7) and the C-terminus (CT) of α_{2B} -AR was conjugated with the N-terminal portion of CD8 glycoprotein and an ICL3 fragment containing the 3R motif (CD8-3R) or the 3A motif (CD8-3A) at the C-terminus. (B) Cell surface expression of CD8-3R and CD8-3A. CD8-3R and CD8-3A were transiently expressed in HEK293 cells and their cell surface expression was measured by flow cytometry following staining using anti-CD8 antibodies in nonpermeabilized cells. * $P < 0.05$ versus CD8-3R (Dong et al., 2012).