Identification and Characterization of Novel β₂AR-Interacting Partners

By

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ABSTRACT

 β_2 -adrenergic receptors (β_2 ARs) are predominantly expressed in cardiomyocytes and regulate cardiac contractility. Prolonged stimulation of these receptors can cause detrimental as well as beneficial effects during heart failure. The ability to transduce these different signals is thought to occur via their association into specific multiprotein complexes containing specific set of heterotrimeric G proteins, effectors, and several other interacting partners that modulate their biosynthesis, signalling, and trafficking. The initial interaction of components comprising these signalling complexes occurs at the level of the endoplasmic reticulum (ER) and at present many of the proteins and processes mediating their assembly remain unknown.

In order to identify these proteins and candidate processes, we developed a novel proteomic approach for β_2AR purification. We validated this approach by detecting several known interacting partners of β_2AR in HEK 293 cells including adenylyl cyclase 3, N-ethylmaleimide-sensitive factor, ubiquitin, Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2) and of course heterotrimeric G proteins. In addition, we identified and confirmed VCP, gp78 and RNF5, proteins related to ER-associated degradation (ERAD), as novel interacting partners of β_2AR . We demonstrated that VCP retrotranslocates β_2AR from ER to the cytosol and the receptor is degraded by the cytosolic proteasome. Proteasome inhibition or VCP knockdown decreased receptor interaction with G $\beta\gamma$ and led to an enhanced signalling response via ERK1/2.

We conclude that ERAD specifically regulates levels of uncomplexed β_2ARs to $G\beta\gamma$ thus has a previously uncharacterized role in quality control of signalling complex assembly. A better understanding of this process might lead to novel therapeutic strategies during clinical conditions such as heart failure where the nature of receptor signalling complexes changes dramatically.

RÉSUMÉ

Les récepteurs β_2 -adrénergiques (β_2ARs) sont exprimés majoritairement dans les cardiomyocytes et régulent la contractilité myocardique. Lors d'insuffisance *cardiaque congestive*, la stimulation prolongée de ces récepteurs peut provoquer des effets bénéfiques mais aussi néfastes. Il est suggéré que les réponses aux différents signaux surviennent suite à l'assemblage de ces récepteurs dans des complexes multiprotéiques. Ces derniers sont composés d'ensembles spécifiques de protéines G hétérotrimériques, d'effecteurs et de plusieurs autres partenaires d'interaction, qui modulent la biosynthèse, la signalisation et l'acheminement membranaire du récepteur. Le premier site d'interaction de ces composants s'effectue au niveau du réticulum endoplasmique (RE). Actuellement, les protéines et les processus de médiation de cet assemblage restent inconnus.

Afin d'identifier ces protéines et ces processus de médiation, nous avons développé une nouvelle approche protéomique suivant la purification du β_2AR . Nous avons validé notre approche en détectant plusieurs partenaires d'interaction connus du β_2AR dans des cellules HEK 293, tels que l'adénylate cyclase 3, la protein sensible de fusion de N-ethylmaleimide (NSF), l'ubiquitine, Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2) et bien sûr les protéines G hétérotrimériques. De plus, nous avons identifié de nouveaux partenaires d'interaction du β_2AR tels que VCP, gp78 et RNF5, des protéines du système de dégradation associé au RE (ERAD). Nous avons démontré que VCP extrait le β_2AR du RE pour le diriger vers le cytosol afin d'être subséquemment dégradé par le protéasome. L'inhibition du protéasome et la

suppression du VCP par l'utilisation de siRNA ont diminué l'interaction du récepteur avec les protéines G et ont augmenté la signalisation du récepteur via la voie ERK1/2.

Nous concluons que le β_2 AR non complexé aux protéines G $\beta\gamma$ est régulé spécifiquement par l'ERAD, ce qui suggère un rôle nouveau pour ERAD dans le contrôle de l'assemblage du complexe de signalisation. Une meilleure compréhension de ce processus pourrait mener à de nouvelles stratégies thérapeutiques utilisées dans des situations cliniques comme l'insuffisance cardiaque congestive, où la nature de la signalisation du récepteur peut changer dramatiquement.

LIST OF ABBREVIATIONS

- ADP adenosine diphosphate
- AKAP A-kinase anchoring protein
- ATP adenosine triphosphate
- Bip binding immunoglobulin protein
- BRET bioluminescence resonance energy transfer
- BSA bovine serum albumin
- CaCl₂ calcium chloride
- cAMP cyclic adenosine monophosphate
- CBP calmodulin-binding protein
- CFTR cystic fibrosis conductance regulator
- CH₃CN acetonitrile
- CHX cyclohexamide
- CMC critical micellar concentration
- CREB CRE binding protein
- CoA coenzyme A
- DABCO -1,4-diazabicyclo[2.2.2]octane
- DDM –*n*-dodecyl-β-D-maltoside
- DMEM Dulbecco's modified Eagle's medium
- DNA -deoxyribonucleic acid
- DTT dithiothreitol
- EDEM ER degradation enhancing α -mannosidase-like lectins
- EDTA ethylenediaminetetraacetic acid
- EGTA ethylene glycol tetraacetic acid
- EPAC exchange protein activated by cAMP

- ER endoplasmic reticulum
- ERAD ER associated degradation
- ERK Extracellular signal-regulated kinase
- ERQC ER quality control
- FASP filter-aided sample preparation
- FP–prostaglandin F2α receptor
- FRET fluorescence resonance energy transfer
- GAPDH glyceraldehyde 3-phosphate dehydrogenase
- GFP green fluorescent protein
- GIRK G protein-activated inward rectifying K⁺ channels
- GPCR G protein-coupled receptors
- GRKs G protein-coupled receptor kinases
- HA hemagglutinin
- HCl hydrochloric acid
- HEK human embryonic kidney
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- His histidine tag
- HRP horseradish peroxidase
- HSP heat shock protein
- IRES internal ribosome entry site
- KCl potassium chloride
- KH₄PO₄ potassium pyrophosphate
- LC MS/MS liquid chromatography-tandem mass spectrometry
- Luc-luciferase
- MAGI 3 membrane-associated guanylate kinase 3

- MAPK mitogen-activated protein kinase
- MgCl₂ magnesium chloride
- MgOAc magnesium acetate
- mRNA messenger ribonucleic acid
- MS mass spectrometry
- Na₂HPO₄ sodium pyrophosphate
- NaCl sodium chloride
- Nedd4 neural precursor cell expressed developmentally down-regulated protein 4
- NEF nucleotide exchange factor
- $NHERF2 Na^{+}/H^{+}$ exchanger regulatory factor 2
- Npl4 nuclear localization factor 4
- OG octylglucoside
- PBS phosphate buffered saline
- PCR polymerase chain reaction
- $PGF2\alpha prostaglandin F2\alpha$
- PI3K phosphatidylinositol 3-kinases
- PKA protein kinase A
- PKC protein kinase C
- Puro puromycin
- $R/G/E Receptor/G \ protein/Effector$
- SBP streptavidin-binding protein
- SDS PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
- TAP tandem affinity purification
- TBS Tris-buffered saline
- TCR T-cell receptor

TEV - tobacco etch virus

- Tr-FRET time-resolved fluorescence resonance energy transfer
- Ufd1 ubiquitin fusion degradation 1
- UPS ubiquitin proteasome system
- VCP-valosin-containing protein
- $\beta_1 AR \beta_1$ -adrenergic receptor
- $\beta_2 AR \beta_2$ -adrenergic receptor
- $\beta_3 AR \beta_3$ -adrenergic receptor
- $\beta AR \beta$ -adrenergic receptor

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INTRODUCTION

G protein-coupled receptors (GPCRs) form the largest family of membrane receptors. They share a distinct architecture composed of 7 α -helical transmembrane domains connected with three extracellular and three intracellular loops, orienting their N-terminus in the extracellular space and their C-terminus in the cytosol. They transduce extracellular stimuli into intracellular signals via activation of heterotrimeric G proteins and subsequently specific sets of effectors. Among the first GPCRs to be characterized by radioligand binding were β -adrenergic receptors (β ARs) [2] and they thus became the prototypical GPCRs used in various studies aimed at understanding receptor signal transduction, modulation and trafficking itineraries.

 β ARs are predominant GPCRs found in cardiomyocytes and their activation by endogenous catecholamines leads to increased cardiac output [3]. Chronic activation of β ARs provides critical ionotropic support that maintains cardiac function during clinical conditions such as heart failure. However prolonged activation also results in detrimental ventricular remodelling and eventually cardiomyocyte apoptosis [4], thus leading to progression of the disease and a worsening of patient prognosis. This is further supported by observations that increased plasma levels of norepinephrine, an endogenous β AR ligand, can be used as an indicator of increased risk of mortality during heart failure [5]. These observations were the basis for the use of β -blockers, antagonists which block the catecholamine binding site of β ARs, in the treatment of heart failure. Catecholamine actions were originally thought to occur through their activation of an eponymous β AR, however it is now known that cardiomyocytes coexpress three subtypes of β ARs, β_1 AR, β_2 AR and β_3 AR, each having a distinct signalling and functional properties [4, 6]. Interestingly, during heart failure only the β_1AR is down regulated, and the role of β_2AR becomes more important [7]. Although activation of both receptors leads to increased ionotropic responses, β_2ARs are thought to activate a wider set of effector molecules. Chronic activation of β_2AR has been shown to provide anti-apoptotic signals in cardiomyocytes, potentially providing beneficial effects during heart failure [4]. Therefore, the use of β -blockers is complicated since β -blockers target the orthosteric binding site of βARs , and the beneficial effects of β_2AR stimulation might be blocked as well. It is important to develop novel therapeutic strategies that will eliminate the detrimental effects of βARs stimulation while retaining beneficial effects mediated by β_2ARs during heart failure. This improved therapeutic strategy certainly requires regulating the activity of specific effector pathways and in order to do so, we need to understand how specific signal transduction events are mediated by GPCRs such as the β_2AR .

Canonical β2AR Signal Transduction

It was initially thought that for GPCRs such as the β ARs, the signal transduction mechanism was composed of essentially three separate components, receptor, heterotrimeric G protein and effectors acting in a linear fashion governed by random and transient collision events, described in the *collision coupling model* [8, 9]. β AR were demonstrated to couple to G α s, which in turn activated a membrane bound enzyme, adenylyl cyclase, enhancing the production of intracellular cAMP, a detectable signalling output in response of the receptor activation [10]. Ligands were thus classified as either agonists or antagonists based on their ability to either induce

or block production of cAMP. The activation mechanism of the receptor was explained by a mechanism where the inactive receptor switches to an active state via conformational changes induced by ligand binding, allowing efficient coupling of receptor to Gasßy heterotrimer, an exchange of GTP for GDP on the Gas subunit, leading to activation of heterotrimeric G proteins and the subsequent activation of effectors such as adenylyl cyclase [11]. The primary signalling outcome of increased cAMP levels is the activation of protein kinase A (PKA) and subsequent phosphorylation of proteins essential for cardiac function such as L-type calcium channels [12, 13], phospholamban [14, 15], troponin I [14], ryanodine receptors [13], myosin binding protein-C [16], and protein phosphatase inhibitor-1 [17], ultimately affecting Ca2+ handling and sensitivity, leading to altered contractility of cardiomyocytes. Later, $\beta_2 ARs$ were observed to be in an active state even in the absence of a ligand, i.e. having basal or constitutive activity, leading to discoveries of ligands that abolish this activity which were termed inverse agonists [18]. Recent studies challenge this simple, linear model of signal transduction by β_2 ARs and add several layers of complexity to their transduction mechanism providing clues regarding how they can regulate several signalling outcomes.

β₂AR Signalling Diversity

Complexity of signal transduction mediated by β_2AR can be appreciated in the signalling pathways themselves, since the receptor can activate several in a cell-type specific manner (reviewed in [19], see Figure 1). The primary wave of signalling, G protein-dependent, does not only involve direct activation of adenylyl cyclase by Gas

subunits, but also involves activation of other signalling pathways by "released" by subunits from activated Gas subunit in cell type-specific manner. It is still not clear whether the heterotrimeric G proteins physically separate into distinct α and $\beta\gamma$ subunits or simply a molecular rearrangement within the subunits to regulate the activity of their effectors [20, 21]. Examples of GBy signalling include activation of G protein-activated inward rectifying K^+ (GIRK or Kir3) channels, necessary for hyperpolarization of cardiomyocytes or neurons [22], and activation of p38 MAPK in HEK 293 cells, thought to regulate cell death [23, 24]. Moreover, β_2 ARs do not only couple to Gas, but can also couple to pertussis toxin-sensitive G proteins, Gai [25] in a PKA-dependent mechanism which activates mitogen-activated protein kinase (MAPK) and to initiate other signalling cascades mediated by $G\beta\gamma$ subunits such as ERK1/2 phosphorylation [26]. Closely related MAPKs ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are key players in cell proliferation, differentiation and apoptosis, and in cardiomyocytes their activation has been linked to cardiac hypertrophy and cardiomyocyte survival [27]. ERK1/2 activation by β_2 AR have been also shown to require activation of cAMP/PKA [28] and c-src [29, 30]. It is likely that the actual mechanisms of ERK1/2 activation and the G proteins coupled to a given receptor are cell-specific.

Following agonist stimulation, the receptor is usually phosphorylated by G protein-coupled receptor kinases (GRKs) which allow recruitment of β -arrestins to the receptor causing physical uncoupling of the activated heterotrimeric G protein from the receptor, effectively terminating the G protein-regulated signal [31]. This interaction is a prerequisite for receptor internalization into clathrin-coated pits which

are pinched off by dynamin into vesicles called endosomes [32, 33], a process known as endocytosis. The internalized receptor can be either degraded via lysosomal enzymes or recycled to the cell surface for another round of activation. The sorting of the receptor to the lysosomes depends on the duration of agonist stimulation [34] and recently has been shown to require ubiquitination of the receptor on specific lysine residues by an E3 ligase, Nedd4, recruited to the receptor in a complex with β -arrestin [35, 36]. It is worth noting that β -arrestins, once interacting with the phosphorylated receptor, act as scaffolding proteins by recruiting other factors involved in activation of a second wave of signalling cascades, which some authors termed "G proteinindependent" but is more aptly described as post G protein signalling. These pathways involve activation of mitogen-activated protein kinases (MAPK) including ERK1/2, p38 MAP, c-Jun N terminal kinase as well as Akt and PI-3K [19]. Furthermore, $\beta_2 AR$ can be phosphorylated by specific isoforms of GRKs, PKA, or protein kinase C (PKCs), depending on the cellular milieu and this also modulates signalling outcomes of the receptor [26, 31, 37]. Thus, $\beta_2 AR$ signalling does not simply turn around a linear signalling cascade leading to a single specific cellular response, rather it involves a complex series of events and a multitude of effectors activated at specific time points, and specific subcellular locations, in a cell type-specific manner.

Realizing that β_2ARs can activate multiple signalling pathways lead to reevaluation of β_2AR agonists, antagonists and inverse agonists since they had mainly been classified based on the one signalling output mediated by adenylyl cyclase. The idea that a ligand can have a different effect on the signalling outcomes generated by a GPCR has been described as "ligand bias" [38, 39], and recent work to determine the ability of $\beta_2 AR$ agonists, antagonists, and inverse agonists to modulate cAMP production or ERK1/2 activation, has allowed a re-classification of ligands as biased. For example, isoproterenol activated both ERK1/2 and cAMP/PKA signalling pathways, on the other hand propranolol, a classical antagonist for the cAMP/PKA pathway could in fact activate the ERK1/2 pathway as a biased partial agonist [40]. This biased signalling has also been thought to potentially mediate beneficial outcomes from the use of carvedilol, a β -blocker, during heart failure. This is thought to occur due to its biased activation of ERK1/2 via a β -arrestin mediated signalling pathway downstream of the $\beta_2 AR$ [41]. Thus developing novel drugs that promote activation of this specific pathway during heart failure might be beneficial.

The ability of a ligand to rapidly and selectively activate one signalling pathway over another suggests that receptors exist in more than one active state. In fact, several techniques such as quantitative mass spectrometry and fluorescent spectroscopy demonstrated that β_2AR activation involves several conformations of the receptor which are stabilized by different ligands [42-45]. These multiple conformations are thought to be possible due to the inherent structural flexibility of the receptor.

β2AR Signalling Complexes

Another factor that mediates the complexity associated with β_2AR signal transduction is the ability of this receptor to form stable complexes with multiple proteins. The cellular milieu is complex and cells can express several GPCRs, G proteins and effectors, which must deliver specific and rapid responses, including

distinct responses generated by a single GPCR. A model where critical components comprising GPCR partners, such as heterotrimeric G proteins and effector molecules form stable signalling complexes has been proposed [46]. Initially the presence of such complexes was observed in *Saccaromyces cerevisiae* and *Drosophila* [47, 48].

 $\beta_2 AR$ is now recognized to be in at least a homodimeric form as a result of experiments utilizing differential epitope tagging, coimmunoprecipitation techniques and by BRET [49, 50]. Moreover, it has been shown to heterodimerize with, α_{1b} -adrenergic receptor [51], $\beta_1 AR$ [52, 53], $\beta_3 AR$ [54], serotonin 4 (5-HT) receptor [55], δ and κ -opioid receptors [56, 57], prostaglandin E receptor 1[58], the bradykinin type 2 receptor [59], angiotensin II type I receptors [60], C-X-C chemokine receptor type 4 receptor [61], oxytocin receptor [62, 63] and the cannabinoid CB1 receptor [64], and the list will certainly expand in the future. Several of these interactions have effects on either trafficking itinerary and/or signalling outcomes and may actually facilitate allosteric interactions between dimer partners.

Further evidence for the existence of larger signalling complexes in mammalian cells began with observations that G proteins can be stably associated with receptors reviewed in [46], including demonstration that β_2AR can be purified, independent of the receptor activation state, with the G α s heterotrimer [65]. These stable interactions have been extensively studied and have been confirmed with techniques utilizing Förster resonance energy transfer in techniques such as BRET and FRET, allowing detection of protein-protein interactions in living cells [66]. Using the FRET approach, the interaction between the G protein subunits and receptors were shown to be specific, for example, the α_2 -adrenergic receptor was shown to interact

with Gai but not Gas and prostaglandin I₂ receptor interacted with Gas but not Gai [67]. Moreover, it has been shown that particular G $\beta\gamma$ subunit combinations also confer specificity to GPCRs. For example, vasoactive intestinal peptide receptors mediate the stimulation of adenylyl cyclase by activating Gas $\beta_2\gamma_2$, while β_2AR stimulates this enzyme by coupling to Gas $\beta_1\gamma_7$ in HEK 293 cells [68]. This suggests that specific sets of heterotrimeric G proteins can be pre-coupled with receptors in order to facilitate specific signal transduction events.

Further, stable interactions of β_2AR and effector molecules such as L-type calcium channels, calcium-activated potassium channels, inward rectifying potassium channels and adenylyl cyclase have been demonstrated using co-immunoprecipitation techniques or BRET assays [69-73], and these interactions were demonstrated to occur independent of the activation state of the receptor [71, 74]. This suggests that the complex components are not recruited as a result of GPCR activation rather they stably interact with each other prior to the activation of the receptor by agonist [74].

In addition, the receptor can interact with cytoskeletal proteins, nuclear proteins, molecular chaperones, scaffolding proteins, and other proteins involved in protein assembly, trafficking and intracellular signalling. These interactions are quite diverse and are related to dynamic life cycle of a given GPCR within a cell and thus can significantly tailor signal transduction in a given cellular context. Specific examples for β_2AR include the PDZ domain-containing protein, NHERF1, which is recruited to the C-terminus of β_2AR once activated, relieves inhibition by this factor on the Na⁺/H⁺ exchanger type 3 [75, 76]. Another example is the receptor's interaction with MAGI-3, which dampens signalling through the ERK1/2 pathway [77]. Other

proteins can mediate GPCR signalling outcomes by acting as scaffolding proteins. Specific, well-studied examples for β_2 AR include the interaction with scaffolding protein AKAP79/150 which tethers PKA close to the receptor, ensuring more rapid and specific phosphorylation of local substrates including the receptor itself. [78].

Several proteins have been shown to regulate GPCR trafficking, either facilitating transport towards the plasma membrane or modulating endocytosis of the receptor following activation. In the case of β_2AR , proteins such as Sar1 and Rab GTPases, which govern specific steps of GPCR trafficking [79], have been shown to impact anterograde β_2AR transport, examples include Sar1 [74] and Rabs 1,2, 6, 8 and 11 [80-83]. Other proteins that bind directly to β_2AR and regulate its internalization and recycling include N-ethylmaleimide sensitive fusion protein and ADP-ribosylation factor 6 [84, 85].

The multitude of β_2 AR interacting partners suggest that the receptor itself acts as a scaffold for formation of specific signalling complexes composed of unique sets of heterotrimeric G protein subunits and effector molecules. Moreover, these complexes can interact with other accessory proteins necessary for modulation of signalling outcomes and trafficking. Recently, in an effort to identify factors necessary for the formation and/or regulation of GPCRs and their signalling complexes, several genomic and proteomic approaches have been developed (reviewed in [86]).

Proteomic Approaches to identify GPCR-interacting proteins

Mass spectrometry (MS) has become a highly sensitive tool used for protein identification. Coupled to biochemical purification techniques, it also allows identification of interacting partners of purified proteins [87]. In order to apply this approach to GPCRs, numerous steps are involved and each step requires optimization tailored specifically for each individual receptor (Figure 2). Prior to performing biochemical purification, the receptor must be extracted from membranes (i.e. solubilized) in its functional form and this is facilitated by detergents.

Detergents are organic molecules that share a common structure composed of a hydrophobic carbon moiety and a polar or charged headgroup. They can be classified based on their head groups into four categories: non-ionic, anionic, cationic and zwitterionic. Non-ionic detergents are often preferred for GPCR solubilization, however the ultimate choice of detergent for GPCR purification should be determined empirically [88]. Depending on their physical properties and concentration, detergents can form micelles, i.e. non-covalent aggregates of detergent monomers, of a defined size in aqueous solutions. The minimal concentration required to form these micelles is defined as critical micellar concentration (CMC). The formation of these micelles is essential for successful solubilization of membrane-bound proteins, since hydrophobic regions of proteins such as the seven transmembrane domains of GPCRs incorporate into these micelles via hydrophobic interactions and in this way, become water soluble [89]. Following solubilization of the membranes, the GPCR along with interacting partners are purified using biochemical purifications.

Although several biochemical methods have been developed (reviewed in [86]), only two methods allow purification of protein complexes associated with intact receptors under native conditions. The classical approach to purify such protein complexes is via receptor immunoprecipitation using antibodies ideally recognizing

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their extracellular domains or epitope tags. Although there are successful examples using specific antibodies to purify and identify protein complexes associated with GPCRs [90], several difficulties arise in producing high quality antibodies against native receptors that meet the required stringency criteria for specificity, affinity, and sensitivity [91, 92], therefore antibodies directed against epitopes of tagged receptors are often used to purify specific receptors and their signalling complexes. Several other advantages of epitope-tagging method over immunoprecipitation with native antibodies include the possibility of standardizing the purification protocol and thus achieve a high-throughput purification technique. Also, overexpressing the protein of interest in a host cell facilitates the purification of low abundance proteins, such as GPCRs and enhances detection of associated proteins. Several epitope tags for immunoaffinity purifications have been developed and this list is quiet extensive and includes tags such as HA, FLAG, c-myc, CD and GFP [93-97]. For example, the use of FLAG affinity purification for identification of protein complexes was first utilized in purifying several protein complexes composing 25% of the yeast proteome by tagging 10% of the yeast predicted proteins [98]. This technique was also applied to several mammalian proteins including GPCRs to demonstrate novel interactions cannabinoid receptor/90kDa heat between shock protein and dopamine receptor/calnexin interactions [99, 100]. Another method recently developed to identify GPCR-interacting partners is tandem affinity purification (TAP). This technique relies on two sequential affinity purification steps using proteins labelled genetically with dual epitope tags. Common tags used for TAP include combinations of the following binding domains: protein A, protein G, Flag, His, streptavidin, and

calmodulin. In fact, using this technique several novel GPCR-interacting partners have been identified [90, 101-103]. The final elution resulting from such biochemical purifications must be then prepared for MS analysis.

There are two major strategies to prepare the final sample for mass spectrometry (MS) analysis: in-gel digestion and gel-free digestion methods. In-gel digestion is the only method currently used for proteomic analysis of GPCRs and their interacting partners. This procedure, initially described in 1996 [104] consists of 1D or 2D electrophoresis of the purified bait protein along with its partners, staining the gel, excising bands/spots of interest, and digestion by proteases such as trypsin. This procedure yields purified peptides ready to be analysed by MS, since running the gel separates proteins in complex mixtures and efficiently removes detergents and other contaminants from samples. However several drawbacks are associated with this procedure which can greatly hinder analysis of the final eluate. Low abundance proteins are often missed due to limited sensitivity of gel staining techniques such as silver or Coomassie staining [104]. Also several difficulties are associated with analysis of membrane proteins with low abundance and due to their membrane spanning segments, these can be difficult to digest effectively with trypsin [105]. Other problems associated with solubilization and separation of membrane proteins during gel electrophoresis can also be encountered [106], thus limiting detection of membrane proteins and low peptide yield of their interactors. These problems severely limit analysis of GPCRs and their corresponding interactors. Moreover, gel spot picking is labour-intensive and requires extensive handling of samples, increasing the

possibility of contamination and loss of material. Therefore another method of sample processing prior to MS analysis is required.

An alternative to in-gel trypsin digestion is in-solution digestion of proteins. This technique allows for digestion of the final eluate (post-purification) without the need to run SDS-PAGE gels, thus ensuring that the entire sample is digested and analyzed. Another added advantage of in-solution digestion is that lower amounts of starting material and trypsin to perform the digestions are required. However this procedure is not used for proteomic analysis of GPCRs since the presence of detergents in the final elution presents a major difficulty in subsequent MS analysis. Detergents can be ionized and create strong signals, thus dominating mass spectra and obscuring analysis of low abundance peptides [89]. Therefore, they must be removed from in-solution digested samples prior to the MS analysis.

To remove detergents several techniques are used including dialysis, adsorption on biobeads, affinity-chromatography, gel filtration and protein precipitation [89]. Detergents with high CMC are often removed by dialysis. The sample is first diluted below the CMC which disrupts micelles into monomers. Dialysis of the diluted sample against detergent-free buffer allows the passage of these monomers through a semipermeable membrane. Dialysis is performed for several days and multiple changes of the buffer are required [107]. Thus this technique demands large amounts of material and is time consuming. Detergents with low CMCs are usually removed by adsorption to hydrophobic beads, biobeads, and column chromatography. Major disadvantages of these techniques include the need for several washing steps during which interacting partners can be lost [107]. Gel filtration is

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another technique that separates detergent micelles from protein-detergent complexes. However, during this procedure the detergent is not completely removed from the sample and they require relatively high protein amounts [108]. These limitations make the in-solution detergent removal challenging during proteomic studies of GPCRs. Recently two novel methods that overcome these difficulties and allow efficient removal of detergents have been described 1) Filter-Aided Sample Preparation (FASP) [109] and 2) ethyl acetate extraction of octylglucoside [110], a detergent commonly used to solubilize GPCRs. Filter-Aided Sample Preparation (FASP) is a method that permits buffer exchange, digestion of the sample and detergent removal in one ultrafiltration device, thus minimizing peptide loss and enhancing detection of low abundance proteins. Although this technique was used to remove SDS, a detergent commonly used to lyse cells, it can be potentially used to remove other types of detergents such as the ones used for GPCR purification. On the other hand, ethylacetate extractions can only be applied to extract octylglucoside; however this technique is easier than FASP and does not require expensive ultrafiltration devices. Nevertheless, both methods can be used to prepare the purified GPCR samples for MS analysis and thus the use of in gel-digestion protocol can be avoided during proteomic analysis of GPCRs and their interacting partners.

In this study we have taken advantage of both of these techniques in order to develop a novel proteomic approach to identify interacting partners of the β_2 AR. As a result we have identified several factors that are related to ER quality control and ER-associated degradation as specific interactors of β_2 AR.

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Biosynthesis of GPCRs

GPCRs are synthesized from mRNA products by ribosomes bound to the ER membrane, as its being translated, the amino acid signal sequence targets, translocates and governs the folding of three-dimensional structure of the GPCRs. Targeting and translocation is regulated by either a cleavable N-terminal signal sequence found only on about 10% of the GPCRs or by the 2nd transmembrane domain in receptors such as β_2 AR [111]. Interestingly, addition of a cleavable signal sequence to the β_2 AR leads to enhanced functional receptor cell surface expression [112]. Correct three dimensional structure results from several non-covalent interactions, covalent interactions and hydrophobic interactions within the receptor's amino acid sequence. The folding of this polytopic membrane protein is monitored by several molecular chaperones and folding factors comprising the primary ER quality control system. These recognize hydrophobic patches and unpaired cysteine residues and, by binding to the unfolded protein, they catalyze folding reactions and/or stabilize the intermediate forms in order to allow more time for the correct structure to form and prevent intermediate forms from aggregating within the ER membrane [113]. Only a few ER-resident chaperones have been demonstrated to interact with GPCRs, these include calreticulin, calnexin and Bip and none have been identified to date to interact with the $\beta_2 AR$ [114-117]. The process of folding is stringent and critical since even single amino acid mutations within a GPCR, can lead to folding difficulties and ER retention. This causes loss of function phenotypes and ultimately human diseases such as retinitis pigmentosa, nephrogenic diabetes insipidus and hypogonadotropic hypogonadism involving rhodopsin, V2 vasopressin and gonadotropin-releasing hormone receptors respectively

[118]. Interestingly folding difficulties are not only associated with mutations in the amino acid sequence but have been also characteristic of wild type GPCRs. Several GPCRs have been shown to maturate inefficiently including δ -opioid receptors [119], gonadotropin receptors [120], luteinizing hormone receptors [121], follicle stimulating hormone receptors [122] and the long isoform form of D2 dopamine receptors [123], suggesting that the acquisition of the appropriate three dimensional structure is a ratelimiting factor for ER export of some GPCRs. To explain this phenomenon it has been proposed that the maintenance of conformational flexibility, necessary for signalling to so many pathways, makes GPCRs more prone to misfold. This increases their residence time in the ER and ultimately they can be removed via ER-associated degradation (ERAD) and the ubiquitin-proteasome system (UPS) [124]. In fact, ERAD has been shown to regulate the levels of several GPCRs including δ -opioid [125], olfactory receptors [117], rhodopsin [126], cannabinoid 1 receptor [127] and the thyrotropin-releasing hormone receptor [128]. Since their retention is dependent on conformational criteria, we can suggest that functional GPCRs can be retained in the ER and subsequently be degraded, thus stabilization of their structure in the ER can therefore promote their functional cell surface expression. This concept was tested by using cell-permeable antagonists of V2 vasopressin receptors, to rescue cell surface expression of intracellularly retained mutant receptors responsible for nephrogenic diabetes insipidus. Incubation with a cell-permeable antagonist resulted in increased functional receptors at the cell surface [129]. Similar experiments were performed by other groups using either agonists, antagonists or chemical chaperones, to rescue cell surface expression of receptors such as gonadotropin releasing hormone receptor mutants and ER-retained rhodopsin mutants [130, 131]. In addition, specific ligands for δ -opioid [132] and gonadotropin-releasing hormone receptors promoted wild type receptor maturation and cell surface expression [133]. The action of these lipid soluble ligands is thought to occur by binding to intracellularly retained GPCRs to promote their correct folding by stabilizing their structure.

ER-associated degradation

In order to limit misfolded proteins retained in the ER, ER quality control systems recognize proteins as being aberrantly folded and target them for degradation. This process involves several stages, including recognition and targeting of misfolded substrates, retrotranslocation to the cytosol and finally degradation by the cytosolic proteasome via the ubiquitin-proteasome system (UPS; Figure 3).

Substrate recognition is poorly understood, especially in the context of targeting GPCRs to ERAD. Several chaperones responsible for the primary quality control system have been demonstrated to play an important role in this step and they include chaperones such as HSP70s [134], HSPs40s [135] and nucleotide exchange factors (NEFs) [136]. Other proteins that are glycosylated at their N-terminus can enter the calnexin/calreticulin cycle where they cycle between binding and release from these chaperones. This process is regulated by two enzymes UDP-glucose-glycoprotein glycosyltransferase and glucosidase II by adding or removing terminal glucose from the N-terminus [137]. ER degradation enhancing α -mannosidase-like lectins (EDEMs) and lectins that contain mannose-6-phosphate receptor-like domains can recognize misfolded glycoproteins and deliver them to the retrotranslocon [138].

The identity of the retrotranslocon is still not well characterized but may involve proteins such as Sec61 [139] and derlins 1-3 [140].

Polyubiquitination serves as a signal for proteasomal degradation and this post-translational modification is accomplished by the sequential action of several enzymes, E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases [141-143]. Several E3 ubiquitin ligases have been found to reside in the ER and are thought to play a role in targeting substrates to ERAD [144]. The first identified and most studied mammalian E3 ligase integral to ER membrane is autocrine motility factor receptor (variously called gp78, RNF45 or AMFR). This protein mediates polyubiquitination preceding retrotranslocation and can interact with several other ERAD factors such as RMA1/RNF5 and p97/valosin containing protein [145, 146]. Knockdown of this E3 ligase had been shown to abolish ERAD for several proteins [147]. Other E3 ligases residents of ER include synoviolin (Hrd1), Trc8, membrane-associated ring finger 6 and RMA1/RNF5, moreover some E3 ligases that regulate ERAD substrates can be cytosolic such as Parkin and CHIP and ubiquitinate their substrates as its being retrotranslocated to the cytoplasm [134]. Interestingly, ubquitination of some proteins destined for ERAD can be performed by several E3 ligases as it was the case for degradation of T-cell receptor (TCR) subunits. This receptor is composed of several subunits and failure to properly oligemerize in the ER leads to degradation of individual subunits such as TCR- α and CD3- δ [148]. Degradation of CD3- δ subunit can be performed by both E3 ligases Hrd1 and gp78 and this is thought to occur via the same E2-conjugating enzyme, UBC7 [149]. Another example is ERAD control of cholera toxin, where both of these E3 ligases

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have been described to form a complex necessary for its degradation [150]. On other hand, degradation of cystic fibrosis conductance regulator mutant (CFTR) has been shown to occur specifically via gp78 rather than Hrd1. Moreover it has been shown that gp78 recognized polyubiquitin already present on the substrate and catalyzes further ubiquitination. The initial ubiquitination of this substrate occurs via the RNF5/RMA1 enzyme [151]. Thus some substrates require sequential action of E3 ligases for ERAD to occur.

Following ubiquitination, most ERAD substrates are retrotranslocated to the cytosol to facilitate degradation by the proteasome. In some cases, the proteasome is sufficient to extract substrates from ER [152]. However, this step is often mediated by p97/valosin containing protein (VCP), an AAA+ ATPase that associates with other factors such as nuclear localization fator 4 (Npl4) and ubiquitin fusion degradation 1 Ufd1 [153]. It has been proposed that the polyubiquitin moiety provides a handle for the complex to initiate ATP-dependent extraction [154]. This factor has been show to associate with the proteasome cap and thus deliver the substrate for degradation [154]. Moreover, increasing evidence demonstrated that there are other factors that facilitate substrate degradation and may associate with VCP and/or proteasome such as deglycosylation and deubiquitination enzymes [155].

Rationale/Hypothesis/Objectives

Several lines of evidence suggest that the assembly of core molecules comprising GPCR signalling complexes, that is receptor, G proteins and effectors (R/G/E) occurs during biosynthesis in the endoplasmic reticulum (ER) and these

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complexes are then trafficked to the plasma membrane [66, 67, 73, 74, 156-158]. Using biochemical and biophysical approaches, our group detected interactions between $\beta_2 AR$ -G β_γ [74], $\beta_2 AR$ -adenylyl cyclase [157] and G β_γ -adenylyl cyclase [157] occurring at the level of ER and that blocking anterograte traffic with dominant negative forms of Rabs and Sars, did not alter these interactions [74, 157]. Moreover, increasing evidence suggests that GPCRs homodimerize and heterodimerize in the ER [159-163], including the $\beta_2 AR$ [50]. It seems that the ER environment plays a critical role in assembly of signalling components into specific complexes necessary for transduction events generated by GPCRs. We believe that these assemblies, containing unique complexes of R/G/E may ultimately be responsible for distinct signalling events. We hypothesize that $\beta_2 AR$ complex assembly is driven by chaperones in the ER and other subcellular compartments. Thus, the main objective of this study is to identify novel interacting partners of $\beta_2 AR$ in the ER and to characterize interactions mediating $\beta_2 AR$ signalling complex assembly with a view towards understanding their physiological role in signalling of the receptor. In order to identify novel interacting partners we first had to develop a proteomic method for $\beta_2 AR$ (briefly mentioned earlier), and thus this became our first objective and presented under section one in results and discussion section of this thesis. In order to establish this strategy we overexpressed the receptors in heterologous system which in turn increased the amount of receptors that reside in the ER at one time, although trapping receptors in the ER would be a useful further strategy, it is not required for our initial screen. Following successful purification and MS analysis we have identified several ER-resident proteins implicated in ERAD as specific interactors of β_2AR . ERAD has been shown to be involved in regulating anterograde trafficking of a number of GPCRs and the investigation of its role in modulating β_2AR trafficking and signalling complex assembly has become the second objective of this study described in section two of results and discussion section of this thesis. Identifying interactors required for specific β_2AR signalling complex assembly and disrupting or promoting these interactions with peptidomimetics or small molecules may eventually be converted into novel therapeutic strategies for cardiovascular diseases and medical conditions such as heart failure.
MATERIALS AND METHODS

Constructs

The TAP- β_2 AR construct was generated by PCR amplification of human β_2 AR from HA- β_2 AR construct (generated in our laboratory and verified by sequencing) using the following primers:

Fwd: 5'-GTAAGAATTCATGGGGGCAACCCGGGAAC-3',

Rvs: 5'-GCTGGATCCTTACAGCAGTGAGTCATTTG -3'.

The resulting PCR fragment was cloned using BamHI and EcoRI into pIRESpuro-GLUE vector [164]. The Flag- β_2 AR construct was generated by PCR using TAP- β_2 AR as template and the following primers. The forward primer contained NheI, Kozak and Flag sites:

5'GCAGCTAGCGCCACCATGGATTATAAGGACGATGACGATAAGATGGGG CAACCCGGGAAC-3' and the reverse primer contained BamHI site

5'-CGTGGATCCTTACAGCAGTGAGTCATTTG-3'. PCR products were digested with NheI/BamHI and subcloned into pIRES-puro vector. This construct was verified by sequencing (Génome Québec, Montréal, QC). Cre-LUC constructs for gene reporter assays were generously provided by Dr. Jana Stankova (Université de Sherbrooke, Québec). The gp78-Flag construct was a gift of Dr. Yihong Ye (National Institute of Diabetes and Digestive Kidney Diseases, Bethesda, MD). RNF5-Flag was obtained from Dr. Jean-Luc Parent (Université de Sherbrooke, Sherbrooke, QC). The VCP-myc construct was obtained from Dr. Ron Kopito (Stanford University, Stanford, CA). Flag-G β 1-5, G α s-EE and HA-G γ 2 were obtained from the UMR cDNA Resource Center (<u>www.cdna.org</u>). Exchange protein activated by cAMP, EPAC, tagged with *Renilla* luciferase at its C-terminus and tagged with citrine GFP at its N-terminus was validated previously [165], and was obtained from Dr. Ali Salahpour (University of Toronto, Ontario). This EPAC construct was used for BRET studies.

Reagents and Antibodies

All reagents were purchased from Sigma-Aldrich (St-Louis, MO), unless stated otherwise. The following antibodies were used in this study with appropriate dilutions: monoclonal anti-HA from raw ascites (Covance purchased from Cedarlane Labs (Hornby, ON, Canada)) 1:3,000, polyclonal Anti-Flag 1:1,000, polyclonal Anti-ERK/p44/42(T202/Y204, Cell Signalling Technology, Denver, MA) 1:1,000, polyclonal anti-Gβ (T-20, Santa Cruz Biotechnology, Santa Cruz, CA) 1:1,000, polyclonal anti-RNF5 (Abcam, Cambridge, MA) 1:1,000, polyclonal anti- β_2 AR (H-73, Santa Cruz Biotechnology, Santa Cruz, CA) 1:200, monoclonal anti-β-tubulin (Invitrogen, Burlington, ON) 1:3000, monoclonal anti-GAPDH (Ambion, Streetsville, ON) 1:20,000, HRP-linked FK2 monoclonal antibody 1:5,000 (Enzo life Sciences, Burlington, ON), HRP-conjugated anti-mouse 1:5000, anti-myc (Covance) 1:3,000, and anti-rabbit secondary antibodies 1:20,000 (Sigma). Polyclonal anti-ERK-CT (used 1:1,000) was generously provided by Dr. Bruce Allen (Montréal Heart Institute, Montréal, QC). VCP siRNA (J-008727-09, VCP) and control siRNA #5 (D-001210-05-05) were purchased from Dharmacon (Lafayette, CO). MG-132 was purchased

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from Calbiochem (San Diego, CA). PGF2α was purchased from Cayman (Ann Arbor, MI).

Cell Culture and Transfection

HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone laboratories, South Logan, UT), penicillin and streptomycin (Wisent) at 37°C with 5% CO₂. Stably expressing TAP- β_2 AR and Flag- β_2 AR in HEK 293 cells were selected with 1 µg/ml of puromycin (InVivoGen, San Diego, CA). HA-FP stably expressing in HEK 293 cells were obtained from Dr. Stéphane Laporte (MUHC Research Center) [166]. Cells plated in 6-well plates or in T75 flasks were transfected at approximate 70% confluency with either 1 µg of total DNA per one well or 5 µg of DNA per one T75 flask using Lipofectamine 2000 as described by the manufacturer. For transfections with siRNA, 30 nM of siRNA was transfected using 5 µl of Lipofectamine 2000 as described by the manufacturer (Invitrogen) per well in a 6-well plate or using 30 µl of Lipofectamine 2000 per T75 flask. For gene reporter assays the transfection reagent used was polyethylenimine (PEI, Polyscience, Warrington, PA). PEI (1mg/ml) was used at 1:3 ratio with the DNA. Experiments were performed after 48 hours in the case of DNA transfections and after 72 hours for the siRNA transfected cells.

Cell Lysis

Total cell lysate: TAP-β₂AR, Flag-β₂AR or HA-FP stably expressing HEK 293 cells were grown in 6 well plates, following transfections and or treatments indicated in the text the cells were washed twice with phosphate buffer saline solution (PBS 1X, 137 mmol NaCl, 2.7 mmol KCl, 10 mmol Na₂HPO₄, 2 mmol KH₄PO₄, pH 7.4) and resuspended in 300 µl of lysis buffer containing (0.5% *n*-dodecyl-β-D-maltoside (DDM), 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, trypsin inhibitor 5 µg/ml, benzamidine 10 µg/ml and leupeptin 5 µg/ml). Cell lysis was allowed to proceed overnight at 4°C with gentle rocking. The next day, cell lysates were clarified with a 8,000 X *g* centrifugation for 30 minutes, following Bio-Rad protein assay (Mississauga, ON) to determine protein concentration, 25 µg of proteins were used for western blot analysis.

Crude Membrane Preparations: One T175 flask of confluent native HEK 293 cells, stably expressing TAP- β_2 AR or Flag- β_2 AR was grown per condition for small scale purifications. Once protocols were optimized, larger scale purifications were performed using 8 T175 flasks either stably expressing TAP- β_2 AR, Flag- β_2 AR or native HEK 293 cells. All steps were performed on ice using chilled solutions. Cells washed on plates with PBS 1X were pelleted by 1,000 X *g* centrifugation for 5 minutes and lysed in 10 ml of lysis buffer (Tris 5mM pH 7.4, EDTA 2mM, protease inhibitor cocktail (trypsin inhibitor 5 µg/ml, benzamidine 10 µg/ml, leupeptin 5 µg/ml). Samples were then homogenized with a Polytron (Ultra Turrax T18 basic, IKA) with 2 bursts for 10 seconds each at 50% maximal setting. Cellular debris was

cleared with 1,000 X g spin and the supernatant was collected from which crude membranes were pelleted by a 30,600 X g centrifugation for 20 minutes.

Subcellular Fractionation: One T75 of native HEK 293 cells, TAP- β_2 AR, Flag- β_2 AR or HA-FP stably expressing HEK 293 cells were grown to 70% confluency when transfected or to 100% confluency when treated as indicated in the text. Cell lysis was followed as for crude membrane preparation with the following modifications. After cell lysis and the cellular debris have been cleared total membranes were pelleted by a 100,000 *x g* centrifugation for 60 minutes. The supernatant was collected and represents the cytosolic fraction. The 100,000 *x g* pellet was washed twice with lysis buffer and resuspended in 500 µl of solubilization buffer containing 0.5% n-dodecyl β -D-maltoside (DDM), 75 mM Tris-HCl pH 8.0, 2 mM EDTA, 5 mM MgCl₂, and the protease inhibitor cocktail mentioned above, overnight at 4° C on a rocker. The next day, insoluble fractions were separated from solubilized membranes by a 20 min spin at 40,000 x g, following protein quantification using the Bradford assay (Bio-Rad), 25 µg of the sample was kept for western blot analysis.

Optimized Tandem Affinity Purification for MS analysis

Small-scale crude membrane preparations were resuspended in 500 μ l-1.0 ml of solubilization buffer containing 0.5% DDM mentioned above. Large-scale crude membrane preparations were resuspended in 8 ml of 0.5% DDM containing solubilization buffer. Resuspended membranes were incubated on a rocker overnight at 4°C. The next day, insoluble fractions were separated from solubilized membranes by a 20 min spin at 40,000 x g. For western blot analysis, 25 μ g of solubilized crude

membranes were kept prior to the purification (Fraction A). The rest of the solubilized membranes were incubated with 100 µl of pre-washed streptavidin-Sepharose beads (GE Healthcare, Uppsala, Sweden) overnight on a rocker at 4°C. After incubating beads with solubilized membranes, the supernatant was collected and 50 µl was kept aside for SDS-PAGE analysis (Fraction B) to determine binding efficiency to the beads. The beads were washed three times with 500 μ l of solubilization buffer followed by 4 washes (250 µl each) with calmodulin binding buffer (50 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.5% DDM, 2 mM CaCl₂). Retained proteins were eluted with 250 µl of streptavidin elution buffer (calmodulin binding buffer plus 10 mM D-Biotin) and 40 µl was subjected to SDS-PAGE analysis to determine elution efficiency (Fraction D). The elution efficiency was verified by washing the beads post-elution and analyzing 50 μ l of this wash by SDS-PAGE (Fraction E). The streptavidin elution was then incubated with pre-washed calmodulin-sepharose beads (GE Healthcare) for 3-4 hours at 4°C with shaking. Beads were then centrifuged for 2 minutes 200 x g, the resulting supernatant was kept and binding efficiency was analyzed by SDS-PAGE (Fraction F), the beads were then washed 3 times with 1 ml calmodulin binding buffer and resuspended in 250 μ l of calmodulin rinsing buffer (50 mM ammonium bicarbonate pH 8.0, 75 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 2 mM CaCl₂, 0.5% DDM). Resuspended beads were transferred to BioRad spin columns (BioRad) placed over a microcentrifuge tube, and another wash was performed with 250 μ l rinsing buffer. Efficiency of tagged receptor binding to calmodulin beads was further analyzed by SDS-PAGE of this wash (Fraction G). The final elution was performed by sequentially applying 100 µl of

calmodulin elution buffer (50 mM ammonium bicarbonate pH 8.0, 25 mM EGTA, 0.5% DDM) to the column and incubating the beads at room temperature for 5 minutes. The eluate was collected following a 200 x g centrifugation. Beads were then washed twice with 100 μ l of calmodulin rinsing buffer. To verify elution efficiency 20 µl of the final elution (Fraction H/I) and 50 µl of the final wash (Fraction J) was analyzed by SDS-PAGE. The rest of the eluted proteins were reduced by incubation with 25 mM DTT for 40 minutes at 55° C followed by alkylation with 100 mM iodoacetamide for 20 minutes at room temperature. Samples were then lyophilized by vacuum centrifuge to a volume of 30 µl. In order to remove detergent and digest eluted proteins prior to MS analysis, samples were processed by filter-aided sample preparation (FASP) as described in [109] with slight modifications. Briefly, lyophilized samples were mixed in Microcon YM-10 (Millipore, Billerica, MA) filter units with 200 µl of 8 M urea in 0.1 M Tris/HCl pH 8.5 and centrifuged for 40 minutes at 14,000 x g followed by another addition of 100 μ l with another cycle of centrifugation. The next two cycles of washes were performed with 100 μ l of 8 M urea in 0.1 M Tris/HCl pH 8.0. The filter unit was then transferred onto a new microcentrifuge tube and retained proteins were digested with addition of 1.2 μ g proteomics grade trypsin diluted in 120 µl of 0.05 M ammonium bicarbonate pH 8.0 to the filter unit and gently shaken for 1 minute. Protein digestion was allowed to proceed at 37° C in a humidified chamber overnight. The following day, filter units were centrifuged 14,000 x g for 40 minutes and the eluates containing digested peptides were collected. To ensure that most of the peptides were collected 50 µl of 0.5 M NaCl was added to the filter unit and the eluate obtained after 14,000 x g

centrifugation for 20 minutes was combined with the first elution. Eluted peptides were then desalted and acidified prior to MS analysis as described [109] using *3M* Empore HP *Extraction disk cartridges* (*C18-SD*) as a result the final elution contained peptides in a 70% CH₃CN solution that was then lyophilized to dryness.

Optimization of FLAG-based Purification for MS Analysis

Small-scale crude membrane preparations were resuspended in 500 μ l-1.0 ml, large-scale crude membrane preparations were resuspended in 8 ml of solubilization buffer containing 1% *n*-octyl- β -D-glucoside ((OG, octylglucoside), 75 mM Tris-HCl pH 8.0, 2 mM EDTA, 5 mM MgCl₂, protease inhibitor cocktail)) of which 40 µl was kept for SDS-PAGE analysis. The remainder of the solubilized membranes were incubated with washed 100 µl of anti-FLAG monoclonal agarose beads overnight at 4° C on a rocker. The next day, beads were washed three times with 500 µl of solubilization buffer and transferred onto Bio-Rad spin columns (BioRad) and washed twice with 100 µl of rinsing buffer (50 mM ammonium bicarbonate (pH 8.0), 75 mM KCl). The beads were incubated twice with elution buffer, 0.1 M glycine pH 3.0 solution, (100 µl) for 5 minutes at room temperature and 20 µl of this elution was analyzed by SDS-PAGE. The rest of eluate was reduced with 25 mM dithiothreitol for 40 min at 60°C and alkylated by treatment with 100 mM iodoacetamide or 20 minutes in the dark. The eluate was then incubated with 4 μ l of 1 μ g/ μ l proteomics grade trypsin stock solution prepared in 0.1 M HCl solution at 37°C overnight. Detergent

was removed from the peptide solution by water-saturated ethyl acetate extraction described in [110]. Purified peptides were lyophilized until dryness.

Mass Spectrometry

The resulting peptide mixture was analyzed by liquid chromatography- tandem MS (LC-MS/MS) using a LTQ-XL Linear Ion Trap Mass spectrometer (Thermo Scientific). The acquired tandem mass spectra were searched against a FASTA file containing the human NCBI sequences using a normalized implementation of SEQUEST running on the Sorcerer platform (Sage-N Research). The resulting peptide identifications were filtered and assembled into protein identifications using peptide and protein prophets (Institute of Systems Biology, Seattle) [167, 168].

Immunoprecipitation and Streptavidin Bead Purification

TAP- β_2 AR, Flag- β_2 AR stably expressing HEK 293 cells or native HEK 293 cells were grown in 1 T75 flask and following either treatments or transfections as indicated in the text, the cells were fractionated as described above. Solubilized membranes and the cytosolic fraction resulting from 100,000 X *g* centrifugation were subjected to protein quantification (Bio-Rad), 25 µg of protein samples were kept for western blot analysis representing the input prior to immunoprecipitation or streptavidin bead purifications. In all cases, 500 µg of solubilized membranes or cytosolic fractions were applied to 40 µl of pre-washed with appropriate buffer anti-FLAG monoclonal agarose beads or to 40 µl of streptavidin-Sepharose beads (GE Healthcare) and incubated overnight at 4°C on a rocker. The next day, the supernatant

was removed and the beads were washed three more times with solubilization buffer (for membranes) or lysis buffer (for cytosolic fractions). In order to elute the proteins from FLAG-monoclonal agarose beads, the beads were washed once with 1X Trisbuffered saline (TBS) solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubated 30 minutes with 100 μ l of elution buffer containing 150 ng/ μ l Flag peptide in 1X TBS, the supernatant obtained was then transferred to new tubes. In the case of streptavidin-Sepharose beads, the elution was performed by adding 100 μ l of 4X loading buffer and heating the sample for 15 minutes at 65°C. 50 μ l of eluates were analyzed by SDS-PAGE and western blot analysis.

Confocal Immunofluorescence Microscopy

Non-permeabilized cells: TAP- β_2 AR, Flag- β_2 AR stably expressing HEK 293 cells or native HEK 293 cells were seeded on glass cover slips and then incubated with a blocking solution containing 20 mM HEPES pH 7.4 and 0.2% BSA diluted in DMEM for one hour at 37°C. Cells were then incubated with anti-Flag polyclonal antibody or monoclonal Anti-HA diluted 1:200 in DMEM for 60 minutes at 37°C. After an hour incubation, cells were washed three times with DMEM warmed to 37°C Cells were then fixed with chilled (4°C) 4% paraformaldehyde for 5 minutes and washed three times with PBS 1X and incubated for one hour with secondary antibody at room temperature with goat anti-mouse or rabbit secondary antibodies conjugated with Alexa Fluor 488 or 555 (Molecular Probes) diluted 1:600 in PBS-BSA 1% solution. After secondary antibody binding the cells were washed three times with

PBS 1X and mounted onto microscope slides with a drop of mounting media containing 0.1% DABCO and glycerol.

Permeabilized cells: Flag-β₂AR stably expressing HEK 293 cells were seeded on glass cover slips 24 hours prior to be transfected or treated as indicated in the text. Post-treatment or 72 hours after transfection, the cells were then washed three times with PBS 1X, fixed with chilled 4% paraformaldehyde and permeabilized with icecold methanol for 10 minutes on ice. Cells were then washed three times with PBS 1X and incubated with PBS-BSA 1% solution to block non-specific interactions for 1 hour at room temperature. After an hour of incubation, cells were incubated with anti- β_2 AR (1:200 dilution in PBS-BSA 1%) for another hour at room temperature. The cells were then washed three times with PBS 1X and incubated for an hour with anti rabbit conjugated secondary antibody to Fluor 488 (Molecular Probes) for 45 minutes at room temperature diluted 1:600 in PBS-BSA 1% after secondary antibody binding the cells were washed three times with PBS 1 X and mounted onto microscope slides with a drop of mounting media containing 0.1% DABCO and glycerol. In both cases, images were collected on a Zeiss LSM-510 Meta laser scanning microscope (Zeiss, Toronto, ON).

Gene Reporter Assays

For gene reporter assays, TAP- β_2 AR, Flag- β_2 AR stably expressing HEK 293 cells were grown to approximately 70% confluency in 6-well plates and transfected with 0.5ug of Cre-Luc reporter constructs using 1.5 µg of PEI per condition. 48 hours later, cells were treated with either 1 µM isoproterenol (100 µM stock solution was

prepared in 100 µM ascorbic acid solution) diluted in DMEM, 0.1 µM ascorbic acid diluted in DMEM or with DMEM for 6 hours. At the end of the treatment, cells were washed twice with PBS 1X, resuspended in 250 µl of lysis buffer (0.1 M potassium phosphate buffer, 1% Triton X-100, 1 mM DTT, 2 mM EDTA) for 15 minutes on a rotator at 4°C. Non-solubilized material was removed by 100,000 x g centrifugation for 5 seconds and cell lysates were collected. Cre-Luc activity was measured using 20 µl of lysate that was distributed into 96-well containing 100 µl of assay buffer (30 mM Tricine, 2 mM ATP, 15 mM MgSO₄, 10 mM DTT, 1 mM D-Luciferin, 1 mM CoA) that was added to each well prior to reading the measurements. Luciferase activity measurements were taken for 30s using a Synergy 2 microplate reader (Biotek). Raw luminescence values were normalized to luminescence values obtained in the untreated condition, resulting in fold-induction values.

Radioligand Binding Assays

To determine levels of the receptor, crude membrane preparations of one T75 flask of confluent TAP- β_2 AR or Flag- β_2 AR cells were resuspended in 500 µl of binding buffer (50 mM Tris-HCl pH 7.4, 3 mM MgCl₂). To assess the levels of the receptor post MG-132 or DMSO treatment as indicated in the text, one T75 flask of confluent Flag- β_2 AR cells was treated with 10 µM of MG-132 diluted in DMEM or with 0.1% DMSO diluted in DMEM for 6 hours, the cells were then lysed followed by cellular fractionation and the obtained pellet post 100,000 X *g* centrifugation was resuspended in 500 µl of binding buffer (50 mM Tris-HCl pH 7.4, 3 mM MgCl₂). In both cases, membranes were homogenized using an all-glass Potter-Elvehjem

homogenizer. 20 μ g of proteins was incubated in 75 mM Tris-HCl pH 7.4, 12.5 mM MgCl₂, 2 mM EDTA binding buffer containing either 10 nM ³H-CGP12177 (PerkinElmer, Waltham, MA) alone or with 0.5 mM alprenolol for 90 minutes at room temperature in a total volume of 500 μ l. At the end of the incubation period membranes were collected on filter paper by filtration using a Brandel cell harvester. Individual filters were then placed in scintillation vials and 4 ml of scintillation liquid was added. Samples were counted for 300 seconds per sample using a liquid scintillation counter (Wallac Winspectral).

Soluble Ligand-Binding Assay

One T75 of TAP- β_2 ARs stably expressing HEK 293 cells were grown to confluency. Cells were washed three times on plates with PBS 1X and were pelleted by 1,000 X *g* centrifugation for 5 minutes and lysed in 10 ml of lysis buffer (Tris 5mM pH 7.4, EDTA 2mM, protease inhibitor cocktail (trypsin inhibitor 5 µg/ml, benzamidine 10 µg/ml, leupeptin 5 µg/ml). Samples were then homogenized with a Polytron (Ultra Turrax T18 basic, IKA) with 2 bursts for 10 seconds each at 50% maximal setting. Cellular debris was cleared with 1,000 X *g* spin and the supernatant was collected from which crude membranes were pelleted by a 30,600 X *g* centrifugation for 20 minutes. Crude membrane preparations were resuspended in 500 µl of binding buffer (50mM Tris-HCl pH 7.4, 3mM MgCl₂). Membranes were homogenized using an all-glass Potter-Elvehjem homogenizer. Following protein quantification using Bradford assay (Bio-Rad), 20 µg of solubilized membranes were incubated with 450,000 cpm equivalent of ¹²⁵I-CYP (PerkinElmer) in the presence or

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absence of 0.5 mM alprenolol in a total volume of 500 μ l of binding buffer (100 mM NaCl, 10 mM Tris-HCl pH7.4, 0.05% of DDM, 2 mM EDTA). Binding was allowed to proceed for 90 minutes at room temperature. Post incubation, samples were applied onto columns containing G-50 Sephadex (pre-packed and equilibrated with binding buffer, GE Healthcare). Columns were eluted six times with 500 μ l of the binding buffer and six individual fractions were collected in counting vials. Radioactivity in different fractions was counted using γ -counter (Packard, Cobra II gamma) for 1 minute.

SDS-PAGE and Western Blotting

Protein samples were diluted in 4X loading buffer (Tris-HCl pH 6.8, 8% SDS, 40% glycerol, bromophenol blue and 5% β-mercaptoethanol) and heated to 65°C for 15 minutes prior to analysis by SDS-PAGE using 10% acrylamide gels. Following electrophoresis, proteins were transferred onto activated PVDF membranes (BioRad). In order to minimize non specific binding, the membranes were blocked with 5% milk dissolved in TBS 1X-0.1% Tween solution for 1 hour at room temperature except when HRP-linked FK2 monoclonal antibody was used in immunoblot analysis, then the blocking was performed with 3% BSA dissolved in TBS 1X -0.1% Tween. The membranes were then incubated overnight at 4°C with the appropriate dilution of primary antibodies as indicated in the *Reagents and Antibodies* section above. Following overnight incubation, the membranes were washed three times with TBS 1X-0.1% Tween solution and incubated with appropriate HRP-coupled secondary antibodies and left to incubate 1 hour at room temperature. Following three washes

with TBS 1X-0.1% Tween solution, the proteins were then visualized with plus ECL (PerkinElmer). In order to assess ERK1/2 activation, total cell lysates were heated to 65° C for 15 minutes and analyzed as described above. Anti-ERK and anti-pERK antibodies were used as previously decribed [52].

MAPK assays

Untransfected HEK 293, stably expressing TAP- β_2 AR, Flag- β_2 AR or HA-FP HEK 293 cells were plated onto 6-well plates at least 24 hours prior to treatments or transfections. Native HEK 293 cells were treated or transfected as indicated in the text prior to MAPK assays. Stably expressing TAP- β_2 AR, Flag- β_2 AR cells or HEK 293 cells post-treatments or transfections, as indicated in the text, were serum starved for one hour and kept at 37° C, after they were treated with 10 µM isoproterenol (stock prepared in 100 µM ascorbic acid solution) diluted in DMEM or with 0.1 µM ascorbic acid diluted in DMEM (vehicle) or with DMEM for 5 minutes. In the case of stably expressing HA-FP HEK 293 cells, these cells were transfected with 30 nM of VCP siRNA or 30 nM control siRNA, 72 hours later the cells were serum starved for an hour and incubated with 1 µM of PGF2 α (prepared in ethanol) diluted in DMEM or with 0.1% ethanol diluted in DMEM for 5 minutes. For all conditions, the cells were then washed twice with PBS 1X and lysed in 200 µl of 4X loading buffer. Lysates were sonicated three times and 40 µl of the lysates was used for SDS-PAGE analysis.

Lance Ultra cAMP Assay

The detection of cAMP levels in HEK 293 cells was performed using the Lance Ultra cAMP detection kit (PerkinElmer) assay as described by the

manufacturer. In this assay, HEK 293 cells were plated in 6-well plates and transfected with 30 nM of VCP siRNA or control siRNA or pre-treated with either 10 µM MG-132 or 0.1% DMSO diluted in DMEM. 72 hours post-transfection or 5 hours after the treatments the cells were resuspended in 4.0 ml of assay buffer from which 5 µl was distributed in 96 well Opti-plate containing 5 µl of various concentrations of isoproterenol (stock prepared in 100 µM ascorbic acid solution), or with 0.100 µM ascorbic acid as control. Treatments were performed in triplicates. The rest of the protocol was performed as described by the manufacturer. Briefly, the cells were incubated at room temperature for 30 minutes and then 5 μ l of the europium chelate (Eu)-cAMP tracer molecule followed by 5 µl of ULight-labeled anti-cAMP monoclonal antibody (mAb) was added per well and incubated for an hour at room temperature. cAMP produced by cells as a result of agonist stimulation competes for binding of the ULight-labeled anti-cAMP monoclonal antibody. Using the Synergy2 plate reader, cells were irradiated with a 340 nm light in order to excite the donor molecule. The excited acceptor molecule via FRET then emits fluorescent signal detectable in Tr-FRET mode. This signal was detected by Synergy2 with 665 nm filer for 10 milliseconds. Tr-FRET obtained in cells treated with vehicle (ascorbic acid) was used as basal level of cAMP activation and response to different agonist concentrations were normalized to that value.

EPAC Assay

HEK 293 cells were plated onto 6-well plates at least 24 hours prior to transfections. The cells were transfected with 3 μ g of EPAC construct and 30 nM of

VCP siRNA or control siRNA using 5 µl of Lipofectamine 2000. 72 hours post transfection, the cells were washed twice with PBS 1X and and resuspended in 500 μ l of PBS 1X. 80 µl of the cell suspension was distributed in 96-well Opti-plate and left to incubate for 2 hours at room temperature. After 2 hours, the cells fluorescence was measured by the Synergy2 reader. Immediately after reading the fluorescence, the cells were treated with 10 μ l of 10X coelenterazine h (final well concentration 50 μ M) and total luminescence and BRET ratios were collected for 5 minutes by Synergy2 reader. Average of these BRET ratios represents basal BRET of the cells. The cells were then treated with 10 µl of 10X isoproterenol prepared in 100 µM of ascorbic acid solution (final well concentration of isoproterenol 10 µM) or with 10 µl of 100 µM ascorbic acid (vehicle condition) and BRET ratios were read for 30 minutes. Upon completion of the assay, the final five BRET readings were averaged and they represent the final average BRET. The net BRET for agonist or vehicle treatment was calculated by subtracting basal BRET from the final average BRET. The Δ BRET for each transfection (VCP siRNA or control siRNA) was then calculated by subtracting the net BRET of agonist from respective net BRET of vehicle.

RESULTS

Section 1: Novel proteomic approach to identity interacting partners of β₂AR

Functional Expression of Flag-B2AR and TAP-B2AR proteins in HEK293 cells

The human β_2AR was first tagged with either the TAP tag (containing calmodulin-binding protein (CBP), an HA-epitope (HA), TEV cleavage site (TEV) and streptavidin binding protein (SBP) tags) or Flag tag at its N-terminus (Figure 4a). Stable cell lines expressing either construct were generated in HEK 293 cells. From this point on, these lines will be referred to as TAP- β_2AR and Flag- β_2AR cells. The TAP- β_2AR construct as well as the stable cell line was created by Darlaine Pétrin in the Hébert lab. The tags were localized at the N-terminus of the receptor in order to ensure that their fusion did not interfere with interactions with intracellular partners which might be necessary for GPCR complex assembly, function and trafficking.

Even at the N-terminus, incorporation of either TAP- or Flag-tags into a receptor could potentially interfere with its trafficking and function. In order to demonstrate proper trafficking and location of the fused receptors, we labelled the stable cells or native HEK 293 cells with anti-HA or anti-Flag antibodies recognizing the tags of the fused constructs and performed confocal immunofluorescence microscopy. We observed specific immunofluorescence on the cell surface of both stably expressing cell lines demonstrating correct localization of the fused receptors, thus neither tag altered receptor trafficking (Figure 4b). No significant immunofluorescence in native HEK 293 was observed (data not shown). The amount

of receptors capable of binding radioligand expressed in the stable cell lines was determined by binding assays with ³H-CGP12177 radioligand on crude membrane preparations were $0.156 \pm 0.039 \text{ pmol/g} (n=3)$ for stable TAP- β_2 AR and $3.470 \pm 0.519 \text{ pmol/mg} (n=3)$ for Flag- β_2 AR. Thus, we would be able to control for the effects of receptor overexpression by using cell lines expressing different receptor levels.

Moreover, we demonstrated that the proper function of the receptors is not compromised by incubating the stable cell lines with β -adrenergic receptor agonist, isoproterenol, which lead to expected activation of adenvlyl cyclase pathway and ERK1/2 phosphorylation. Activation of adenylyl cyclase was demonstrated by induction of cAMP response element (CRE)-dependent luciferase reporter (CRE-Luc) transciently expressed in these cell lines (Figure 4c) and in native HEK 293 cells (data not shown). Activation of CRE-Luc by β_2AR occurs via activation of Gas which activates adenylyl cyclase (AC) resulting in cAMP production. Elevated levels of cAMP in turn activate PKA which phosphorylates CRE binding protein (CREB) at serine-133 [169, 170]. Phosphorylation of CREB permits its interaction with CREBbinding protein and p300 which interact with the basal transcriptional apparatus to initiate transcription of various genes that contain cAMP response element (CRE) in their promoter regions [169]. Isoproterenol treatment induced no response in native HEK 293 cells (data not shown), a 2 fold increase in activity compared to untreated conditions in Flag- β_2 AR stable cell lines and a 10 fold increase in TAP- β_2 AR stable cell lines (Figure 1c upper panel and lower panel). The elevated Cre-Luc activity in TAP- β_2 AR cells compared to Flag- β_2 AR cells can be explained by the different expression levels of the tagged receptors. $\beta_2 AR$ is known to have a basal activity, in

other words the receptor can activate signal transduction pathways in the absence of agonist stimulation [171]. Thus we might expect basal activation of Cre-Luc gene reporter in untreated conditions. It was previously demonstrated that an increased receptor density linearly correlates with an increased basal activity of adenylyl cyclase and production of cAMP [172]. Since the expression level of the receptor in Flag- β_2 AR cells is greater than that of TAP- β_2 AR cells we would expect an elevated basal activity of Cre-Luc gene reporter in these cells, since we normalized the raw data obtained as a result of treatments to the raw data obtained in untreated cells, we then should expect a decreased Cre-luc fold induction as a result of isoproterenol treatment in Flag- β_2 AR cells compared to TAP- β_2 AR. To ensure that the activation of Cre-luc was caused by isoproterenol treatments, we included a negative control in which we pre-treated both stable cells with ascorbic acid solution, a vehicle used for isoproterenol treatments. This treatment had no effect on Cre-luc activity in either stable cell line (Figure 4c). Although this data demonstrated that the overexpressed receptors in both stable cell lines are able to activate adenylyl cyclase in response to isoproterenol treatment, we cannot determine the exact proportion of overexpressed receptors that were in fact active. We cannot therefore say with absolute certainty that all of the interacting proteins only interact with correctly folded receptors but since we would ultimately use our approach to identify compartment-specific interactors throughout the receptor lifecycle, those that do interact with unfolded or inactive receptors would be equally important.

We next assayed the ability of both stably expressing receptors to activate ERK1/2 MAPK. β_2AR is known to activate ERK1/2 in HEK 293 by several transduction

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mechanisms [28, 40, 52, 77, 173]. We demonstrated activation of ERK1/2 as a result of isoproterenol treatment in both stable cell lines by immunoblotting for phosphorylated ERK1/2 (Figure 4d). The phosphorylated ERK1/2 pattern observed as a result of isoproterenol treatment in both stable cell lines was similar to the positive control, HA- β_2 AR transiently transfected in HEK 293 cells (experiments with HA- β_2 AR and TAP- β_2 AR cells were performed by Darlaine Pétrin). Ascorbic acid treatment was used as negative control and no significant activation of ERK1/2 was detected in this case. It should be noted that the levels of phosphorylated ERK1/2 does not correspond to the expression levels of the receptor in either stable cell line and thus these levels cannot be used as quantitative data, this data can only be used to determine whether ERK is activated in response to receptor inactivation.

In addition, we noticed Flag- β_2AR cells had a more sustained ERK1/2 phosphorylation compared to the TAP- β_2AR and HA- β_2AR transciently transfected cells and this could once again be explained by elevated expression level of the receptor in these cells. In summary these results demonstrate that the TAP or Flag tags did not interfere with proper function, structure and location of fused β_2AR in stably expressing HEK 293 cell lines.

Work Flow for $\beta_2 AR$ purification suitable for MS analysis

Detergent Selection

Prior to purification, receptors must be extracted from membranes using detergents. The selection of detergent is critical for successful purification of

signalling complexes since soluble receptors must remain correctly folded and retain a functional state(s) able to interact with other proteins comprising their signalling networks [89]. A non-ionic detergent, *n*-octyl- β -D-glucoside (OG), was particularly attractive due to its ability to preserve functional properties of purified β_2 -adrenergic receptors [174, 175] and more importantly it could be completely removed by ethyl acetate extraction [110]. Unfortunately OG was not compatible with TAP since TAP- β_2 AR receptors solubilized in this detergent were not able to efficiently bind streptavidin beads (data not shown), therefore it was only used to generate solubilized membrane preparations from Flag- β_2 AR cells and perform subsequent FLAG purifications. As an alternative detergent for the TAP protocol, we decided to use N-Dodecyl-β-D-maltoside (DDM) since it is another mild non-ionic detergent known to preserve βAR binding and was previously used in TAP to purify signalling protein complexes associated with GPCRs [176]. Prior to using this detergent in our TAP protocol, we verified that solubilized TAP- β_2 AR could still bind ligand by incubating the DDM-solubilized crude membrane preparations of either native HEK 293 cells or TAP- β_2 AR cells with saturating concentrations of ¹²⁵I-CYP radioligand in a soluble radioligand binding assay. G-50 sephadex-column filtration was used to separate free ¹²⁵I-CYP ligand from ¹²⁵I-CYP bound to solubilized receptor complexes. Specific β adrenergic receptor binding was observed in fractions II and III of TAP- β_2 AR solubilized membranes (Figure 5a) as opposed to native HEK 293 cell membranes (Figure 5b) in which the free ligand was eluted in later fractions. Specific binding indicated that the integrity of $\beta_2 AR$ structure was conserved during membrane solubilization with DDM and therefore this detergent was used for subsequent TAP.

Purification of TAP-\beta_2 AR signalling complexes

The work flow for tandem affinity purification of the TAP- β_2 AR is shown schematically in Figure 6a. The solubilized membranes of TAP- β_2 AR were subjected to two step affinity purification. The efficiency of $\beta_2 AR$ purification was monitored by western blot analysis. The receptor was visualized using anti-HA antibodies and was seen as a sharp band at around 65 kDa as expected taking account of the 12 kDa tag. To perform TAP, solubilized membranes of TAP- β_2 AR cells were first prepared using 0.5% DDM buffer (Figure 6b, lane a) and the solubilized membrane proteins were incubated with streptavidin-agarose beads, following washes that remove most of the contaminants, the receptor streptavidin-agarose bead interaction was competed out by biotin-containing buffer. The receptor efficiently interacted with the beads during this first step of the protocol, as it was absent from the input (solubilized membranes) post streptavidin-agarose beads and in the washes, monitored by western blot analysis (Figure 6b, lanes 2-3). The receptor was fully recovered during the first elution (Figure 6b, lanes 4-5) and also efficiently bound and eluted from the second affinity purification step using calmodulin-sepharose beads (Figure 6b, lane 6-7). The detergent was kept throughout the purification procedures in order to maintain structural integrity of the GPCR as well as interactions with other partners. Maintenance of GPCR-protein interactions throughout the purification procedure was demonstrated by immunobloting for G β subunits, known interactors of the $\beta_2 AR$ [74] in the final elution (from calmodulin beads) following TAP. Transfected G β 1, 3 and 4 subunits tagged with Flag, as well as endogenous $G\beta$ co-purified with the receptor following TAP but were not detected under control conditions where native HEK 293 cells were also transfected with the same set of G β subunits (Figure 6c). Copurification of endogenous G β with β_2 AR demonstrated that our TAP procedure is gentle enough to preserve known interactions yet is stringent enough to remove contaminants efficiently since we were not able to detect G β in the control conditions.

After having validated the TAP protocol, we used a scaled-up crude membrane preparation protocol in order to purify sufficient amounts of the receptor and identify less abundant β_2AR interacting partners. The obtained solubilized membrane proteins were used for the TAP protocol and the eluted proteins from calmodulin-sepharose beads were subjected to filter-aided sample preparation (FASP), one of the gel-free methods compatible with TAP. This technique allows for complete buffer exchange and trypsinization in Microcon YM-10 concentrators, resulting in detergent-free peptides. To desalt peptides, 3M Empore HP Extraction disk cartridges were used to yield peptides suitable for MS analysis (see Figure 7 for schematic representation and addition details of this technique). Performing the LC-MS/MS, we were able to identify 250 and 183 interacting proteins in two separate purification procedures respectively. 97 proteins were identified as common interactors since they were present in both purifications. Classification of these proteins according to function and location demonstrates that the β_2 AR interacts with a diverse set of proteins (Figure 8). Among identified proteins known $\beta_2 AR$ interacting partners, N-ethylmaleimidesensitive factor [84], ubiquitin [177], Gai3[26] and NHERF2 [75] were present (Table 1a), demonstrating that our protocol is successful in identifying known proteins associated with functional $\beta_2 AR$. This data validates our proteomic strategy. Surprisingly, endogenous GBy subunits were not detected in our MS screen despite the fact that we were able to detect them by western blot analysis suggesting that there was loss of material during the detergent removal technique or not all proteins were properly denatured and trypsinized.

Overall, using TAP coupled to MS, we were able to purify functionally relevant interactors associated to β_2 ARs under native conditions, however several known interactors were absent in our MS screens. This may be due to sample preparation prior to MS analysis by FASP. Thus, we proceeded to develop a second FLAG-based immunopurification protocol which has several distinct features compared to TAP in order to improve yields.

Purification of Flag- β_2AR *signalling complexes*

The Flag purification procedure (outlined schematically in Figure 9a) was performed on solubilized Flag- β_2 AR membranes in buffer containing 1.0% octyglucoside to solubilize membranes. The purification procedure was again monitored by western blot analysis and the receptor was detected using anti-Flag antibodies. Specific receptor bands were detectable with a prominent band at 75 kDa, in the input condition representing the solubilized crude membrane preparation prior to FLAG purification (Figure 9b). Although the expected size of the receptor is 46 kDa, the different bands may represent complex, oligomeric structures of receptors [178], moreover glycosylation can affect the molecular weight of β ARs by as much as 11 kDA [179]. In order to determine the identity of this receptor band, further assays could be performed such as enzymatic treatments to directly assess the glycosylation pattern of the receptor. The elution of the sample was performed by exposure to a gentle acid solution (0.1M glycine pH 3.0), and this greatly reduces the amount of eluted. This is normally a major contaminant present after antibody immunoprecipitation experiments but we did not detect any contamination arising from the antibody in our MS screen. We detected both monomeric and oligomeric forms of the receptor in the final elution after FLAG purification (Figure 9b). Interestingly, it seems that there are more oligometric receptors being purified as a result of the FLAG purification. Since we have used total pool of receptors for the FLAG purification the interacting partners identified post mass spectrometry analysis will be representative of a mixed state receptor population. It would be interesting to separate different states of receptors, oligomeric from monomeric, prior to perform the FLAG purifications in order to identify different populations of interacting partners associated to these states. Perhaps, the dimerization status changes between TAP- and Flag-tagged receptors affects the pool of interactors and more careful curation of individual data sets might lead to insights in this regard. We also confirmed the presence of endogenous $G\beta$ in the final eluate from the Flag-agarose beads (Figure 9b) demonstrating that we were able to purify functionally relevant β_2AR interactors following FLAG purification as well.

In order to ensure detection of less abundant proteins we used scaled-up FLAG purification protocol. The final elution from the FLAG beads was then subjected to insolution trypsin digestion and another gel-free method distinct from the procedure used during TAP to remove the detergent. Octylglucoside present in the peptide solution was removed by water-saturated ethyl acetate extractions and the samples were then ready to be analyzed by MS analysis (procedure schematically represented in Figure 10). Performing the LC-MS/MS analysis we were able to identify 366 interacting proteins in the first purification attempt and 257 interacting proteins complexed with the receptor in a second independent purification procedure. There were 177 commonly identified proteins, almost double the number identified by TAP. We repeated the same analysis as for the TAP procedure and generated maps of proteins classified according to location and function (Figure 11). The overall distribution of proteins identified was not significantly different from TAP, once again demonstrating the ability of β_2AR to interact with different partners throughout their life cycle. Moreover, with this procedure we were able to identify adenylyl cyclase 3 and importantly all there subunits that comprise the heterotrimeric G proteins, in addition to the same known interacting partners demonstrated by TAP (Table 2). These results validate the FLAG purification as well as the subsequent preparation of the sample prior to MS analysis.

Section 2: Characterization of novel interacting proteins associated to $\beta_2 AR$ in the ER

Identification of proteins involved in ER quality control and degradation as interacting partners of $\beta_2 AR$

Since we were interested in identifying novel interacting partners of $\beta_2 AR$ in the ER, we focused on proteins classified to this subcellular compartment using Gene Ontology. In the context of a heterologous expression system, we expect receptors to

be detected in intracellular compartments, as well as at the plasma membrane. We were able to identify several proteins, listed in Table 3, which were previously reported to be implicated in either ER quality control (ERQC) and ER-associated degradation (ERAD) in at least two independent purifications by MS analysis. Proteins such as heat shock 70 kDa, DnaJ and calnexin (noted here as a β_2AR interactor, for the first time) have been linked to substrate recognition during ERAD [134, 136, 137, 180]. Autocrine motility factor receptor (gp78) and ring finger protein 5 (RNF5) are E3 ligases integral to the ER involved in ubiquitination of substrates [181]. Sec61 protein is thought to be the retrotranslocon that functions as a channel to deliver ERAD-targeted proteins to the cytosol [139]. We also identified three factors, Ufd1, Npl4, VCP comprising the retrotranslocation machinery [182]. In addition, we identified one of the proteasome subunits and its accessory factor ECM29 which assists in degradation of substrates [180]. ERAD, as discussed in the introduction, is a cellular process involved in regulation of misfolded or misassembled proteins. However several studies have demonstrated that correctly folded proteins, estimated to be as high as 30% of the cellular total, including several GPCRs, are also degraded by this route [183]. Our experiments were performed with wild type $\beta_2 AR$ containing no mutations, and in the case of the TAP-tagged receptor, at physiological levels. Several ERAD-associated proteins were identified in our screens, leading us to hypothesize that wild type $\beta_2 AR$ is also regulated by ERAD. This novel regulation of receptor trafficking and/or assembly might have implications for signalling outcomes generated by this receptor.

Regulation of TAP-β₂AR levels by proteasomal degradation in HEK 293 cells

Proteins subjected to ERAD are degraded by the ubiquitin-proteasome system (UPS). In order to determine if $\beta_2 AR$ was a substrate for proteasomal degradation due to its interaction with ERAD, we evaluated TAP- β_2 AR stability in HEK 293 cells (Figure 12a). In the presence of cycloheximide, a protein synthesis inhibitor, TAP- β_2 AR was degraded within 2 hours. Proteasonal degradation is not the only degradation pathway mediating cellular GPCR turnover. Another major pathway involves lysosomal degradation of receptors [184]. In order to demonstrate that $\beta_2 AR$ turnover (or possibly folding and assembly) was regulated specifically by the ubiquitin-proteasome pathway, cycloheximide treatment was repeated in the presence or absence of the proteasome inhibitor, MG-132, or the lysosome inhibitor, NH_4Cl , as well as vehicle, DMSO. Total cell lysates were then subjected to western blot analysis. Levels of TAP- β_2 AR were recovered, even up-regulated from basal levels, with MG-132 as opposed to NH_4Cl treatment indicating an agonist-independent degradation of TAP- β_2 AR in HEK 293 cells via the proteasome. As a control, we used a stable HEK 293 cell line expressing the human prostaglandin F2 α receptor also tagged with an HA epitope (HA-FP) [166] to determine if the effect of the β_2 AR was specific. HA-FP was not regulated by proteasomal degradation but rather by lysosmal degradation as noted by the marked increase of receptor levels when NH₄Cl was used in conjunction with cycloheximide (Figure 12b).

Subcellular localization of β₂AR following proteasome inhibition

Knowing that $\beta_2 AR$ is degraded by the UPS, we decided to probe the distribution of receptors rescued by treatment with MG-132 in the membrane and cytosolic fractions. Despite the fact that we had validated the functionality of TAP- β_2 AR, to eliminate the possibility that the larger TAP-tag fused to the receptor might have affected β_2AR folding efficiency within the ER and thus trigger the ERAD response in HEK 293 cells, we decided to perform the next set of experiments using Flag- β_2 AR. Flag- β_2 AR stable cells were treated with MG-132 or DMSO, cells were lysed and the cell lysates were fractionated by a 100,000 x g centrifugation to separate membrane and cytosolic fractions. To demonstrate an effective fractionation, we determined the presence of Na^+/K^+ ATPase, a canonical membrane marker, and β tubulin, a cytosolic protein by western blot. We noted the absence of specific bands corresponding to Na^+/K^+ ATPase in the cytosolic fraction or β -tubulin in the membrane fractions, attesting the efficacy of subcellular fractionation (Figure 13a). Solubilized membranes and cytosolic fractions were then subjected to Flag immunoprecipitation and western blot analysis to detect levels of the receptor (Figure 13b top panel). The membrane fractions under conditions of proteasome inhibition contained increased levels of receptors that underwent complex glycosylation in the Golgi (70 kDa marked with **, [185]) as well as immature (46-50 kDa, marked *) forms of $\beta_2 AR$ as compared to the vehicle treated condition visible in both input conditions (prior to immunoprecipitation, data not shown) and as a result of immunoprecipitation (Figure 13b top panel). To confirm the increase in receptor

levels in the membrane fraction, we performed radioligand binding assays, which showed that Flag- β_2 AR pre-treated with vehicle was expressed at 3.470 ± 0.5193 pmol/mg of membrane proteins (n=3), whereas in cells treated with MG-132 the amount of receptors increased to 5.090 ± 0.600 pmol/mg (n=3) of membrane proteins.

We also detected, an immature Flag- β_2AR present in the ER corresponding to 45-49 kDa [185] in the cytosolic fraction following immunoprecipitation (Figure 13b top panel marked with *), confirming dislocation of β_2AR from membranes into the cytosol prior to degradation by the proteasome. To ensure that this effect, retrotranslocation, we observed were due to proteasome inhibition specifically, we again used another selective proteasomal inhibitor epoxomicin in similar experiments [186]. Both proteasomal inhibitors, MG-132 and epoxomicin (Figure 13c), resulted in accumulation of TAP- β_2AR in the cytosol. In contrast, HA-FP was not translocated to the cytosol under these conditions (Figure 13c), again highlighting the specificity of the effect for the β_2AR . Taken together, these results indicate that basal levels of β_2AR in HEK 293 cells are regulated by cytosolic proteasome and the immature form of the receptor in the ER is retrotranslocated to the cytosol prior to being degraded by the proteosome.

Effect of proteasomal inhibition on receptor ubiquitination and GB interactions

Proteins targeted for degradation by the 26S proteasome are often covalently modified by an attachment of at least four ubiquitins [142], therefore we assessed the ubiquitination status of Flag- β_2 AR in the presence or absence of proteasome

inhibition. We immunoblotted endogenous ubiquitin using an HRP-linked FK2 monoclonal antibody, which recognizes both mono- and polyubiquitinylated protein conjugates, the immunoprecipitated Flag- β_2 AR obtained as a result of experiment in Figure 13b. A large smear co-immunoprecipitating with solubilized membrane Flag- β_2 AR was detected following proteasome inhibition (Figure 13b middle panel). This smear is characteristic of proteins that are polyubiquitinated indicating that Flag- β_2 AR is likely to be polyubiquitinated. Interestingly, a similar smear of bands was co-immunoprecipitated with the cytosolic form of the receptor (Figure 13b middle panel). Unfortunately, the Flag-IP was performed after lysis in non-denaturing conditions and thus we cannot conclude that β_2 AR was directly ubiquitinated- this could be assessed by mass spectrometry analysis.

We then decided to probe the effect of proteasome inhibition on receptor interactions with one of its interacting proteins, endogenous G β subunits by immunoblotting the final elutions of experiment described in Figure 13b, for endogenous G β using anti-G β antibodies (Figure 13b bottom panel). Although the amount of immunoprecipitated Flag- β_2AR from membrane fraction was increased with MG-132-treatment (Figure 13b top panel), the amount of G β subunits coimmunoprecipitated with these receptors did not change compared to DMSO-treated cells (Figure 13b bottom panel). This likely indicates a decreased association of Flag- β_2AR receptors with G β subunits under proteasomal inhibition, suggesting that uncomplexed receptors are retro-translocated from the ER to the cytosol.

Effect of proteasomal inhibition on cell surface expression of Flag-β₂AR

Interestingly the levels of β_2AR that underwent complex glycosylation in the Golgi, represented by the 70 kDa band in Figure 13b top panel indicated by **, were also increased due to MG-132 treatment, suggesting that proteasome inhibition enhances ER export and maturation of the receptor. To further test this idea, we performed confocal immunofluorescence experiments on permeabilized Flag- β_2AR stable cells pre-treated with either MG-132 or DMSO (Figure 14) in order to determine the location of Flag- β_2AR . Increased cell surface fluorescence was observed following pre-treatment of cells with MG-132 compared to DMSO, moreover no significant immunofluorescence inside the cells was detected. Consistent with western blot analysis in Figure 13b, these data demonstrate that proteasome inhibition leads to increased amounts of mature Flag- β_2AR able to properly traffic to the plasma membrane. The ability of these receptors to reach the cell surface permitted us to test their functionality.

Effect of proteasomal inhibition on the functional expression and intracellular signalling of $\beta_2 AR$

The ligand binding experiment demonstrated that the increased receptors retained their normal topology. To further assess the functionality of cell surface reaching β_2AR after proteasome inhibition, we assayed ligand-induced intracellular signalling of β_2AR in HEK 293 cells. We pre-treated untransfected HEK 293 cells

with either MG-132 or DMSO and after treatment with different doses of isoproterenol, used time-resolved FRET (Tr-FRET) between fluorescently labelled antibody and labelled exogenous cAMP. cAMP produced by a cell competes with the labelled exogenous cAMP and therefore a decrease in Tr-FRET can be observed. We expressed our results as normalized values of Tr-FRET over the control conditions wher the cells were treated with vehicle. Inhibition of the proteasome did not alter the ability of receptors to signal via the adenylyl cyclase pathway since the dose-response curve generated in response to isoproterenol were superimposable between MG-132 or DMSO pre-treated HEK 293 cells, demonstrating that MG-132 treatment did not enhance the ability of the receptors to signal via the adenylyl cyclase pathway (Figure 15a). We next tested another prominent signalling pathway activated by $\beta_2 AR$, ERK1/2 MAP kinase. Here, we pre-treated HEK 293 cells with either MG-132 or DMSO, followed again by stimulation of endogenous β_2AR with isoproterenol or vehicle for 5 minutes. Cell lysates were then subjected to western blot analysis. Interestingly, basal levels of pERK1/2 were enhanced with MG-132 treatment as opposed to DMSO (Figure 15b). Moreover, pERK1/2 response to stimulation by isoproterenol was enhanced in MG-132 condition compared to DMSO treated cells, demonstrating that proteasomal inhibition enhances MAPK signalling in HEK 293 cells by β_2 AR. Levels of total ERK were not altered by MG-132 treatment although changes in other components of the ERK cascade might account for our observations. Overall, this data indicates that proteasome inhibition leads to increased expression of functional $\beta_2 AR$, however signalling outcomes generated by $\beta_2 AR$ under proteasomal

inhibition are altered depending on the pathway examined with increased ERK1/2 phosphorylation and no change in adenylyl cyclase activation.

VCP interacts with $\beta_2 AR$

We were able to demonstrate that proteasome degradation plays a critical role in β_2AR turn-over, occurring through the ERAD pathway. However receptor ubiquitination has been previously reported to play a role in receptor endocytosis [177]. In these studies, inhibition of the proteasome lead to decreased receptor endocytosis and this could potentially compromise our previous findings. However, our proteomics screen clearly indicated interaction with ER-localized components. In order to validate our MS screen and confirm that receptor synthesis is regulated by the ERAD pathway, we decided to characterize β_2AR interactions with VCP, a protein identified as $\beta_2 AR$ interactor (Table 3). This candidate was of a particular interest to us since its implication in ERAD has been extensively studied [146, 182, 187, 188]. VCP is an AAA⁺ ATPase and its function has been demonstrated to be critical for translocation of ubiquitinated proteins destined for degradation by the proteasome [182]. In order to confirm this interaction with β_2AR , we performed coimmunoprecipitation assay. We overexpressed VCP-myc in stable Flag- β_2 AR cell lines (Figure 16) and immunoprecipitated Flag- β_2 AR (Figure 16 marked with *,**,***, representing several forms of the receptor). Immunoblotting with anti-myc revealed a specific band between the 95 and 130 kDa markers (Figure 16 marked with arrow), corresponding to expected molecular weight of VCP, 97 kDa [189], demonstrating co-immunoprecipitation of VCP-myc with Flag- β_2 AR. These results confirmed VCP, a critical factor for ERAD as an interacting partner of β_2 AR in HEK 293 cells, moreover these results validated our MS analysis of the Flag and TAP pulldowns.

Role of VCP in regulation of β_2AR levels in HEK293 cells

We hypothesized that VCP might be involved in delivering β_2AR , present in the ER membrane, to the proteasome by translocating it to the cytosol in HEK 293 cells. To test this hypothesis we decided to either knockdown endogenous VCP protein or overexpress it in HEK 293 cells and immunoblot for β_2AR levels in these cells. Using siRNA for VCP we were able to engender substantial knockdown of VCP-myc at the protein level in TAP- β_2AR stably expressing HEK 293 cells demonstrated by immunoblotting myc in total cell lysates post siRNA transfection compared to siRNA control (Figure 17a, top panel). Immunoblotting for HA indicated increased β_2AR levels in TAP- β_2AR stably expressing HEK 293 cells compared to siRNA control conditions suggesting that VCP plays a critical role in turn-over of the β_2AR in HEK 293 cells (Figure 17a, middle panel). This effect was again specific to the β_2AR , since the levels of stably expressing HA-FP were not altered by VCP knockdown (Figure 17b).

To demonstrate that VCP acts as a retrotranslocator of β_2AR by delivering the receptor to the proteasome we treated Flag- β_2AR stable cells with MG-132 or DMSO while overexpressing VCP-myc protein or vector control (pcDNA3.1). Cytosolic

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fractions of cell lysates were collected and levels of Flag- β_2AR were detected using anti-Flag antibody. As previously seen under proteasomal inhibiton the immature form of the receptor was detected, faint band at 46 kDa, in the cytosol and levels of this form of the receptor were increased with overexpression of VCP-myc (Figure 17c noted with *). This data demonstrates that β_2AR levels in HEK 293 cells are regulated by VCP which retrotranslocates the β_2AR to the cytosol for proteasomal degradation.

Effect of VCP knockdown on distribution of β_2AR species as well as ubiquitin and G β interactions

To determine the effect of knocking down VCP on the subcellular distribution of Flag- β_2 AR, we transfected VCP or control siRNA into Flag- β_2 AR stable cells, then separated membranes from cytosol and the fractionated cell lysates were then subjected Flag immunoprecipitation. We probed the to lysates preimmunoprecipitation (input condition) and post-immunoprecipitation for Flag- β_2 AR using anti-Flag antibody (Figure 18 top panel). The amount of Flag- β_2 AR was increased in membrane fraction when VCP was knocked down, evident in the input conditions (Figure 18 top left panel representing the input condition). It should be noted that this increase is not represented in the immunoprecipitated condition (Figure 18 top right panel) and can be due to saturation of the Flag agarose beads during immunoprecipitation and does not represent the amount of receptors in the total lysates. Interestingly several forms of the receptor were increased in the membrane fraction including the 46 kDa immature form (noted with *), the 70 kDa glycosylated form (noted with **), as well as receptor oligomers (noted with ***) compared to the control condition (Figure 18 top panel). As expected, there were no cytosolic immature receptors present, consistent with the idea that VCP retrotranslocates this species of β_2AR . (Figure 18 top panel cytosol conditions), rather this species could be detected in the solubilized membrane fraction, since we detected an increased levels of the immature receptor in the solubilized membrane fraction under VCP knockdown compared to control (Figure 18 marked with * left panel). This demonstrates that VCP knockdown inhibits retrotranslocation of immature β_2ARs from the ER, trapping them in this subcompartment. We also studied receptor ubiquitination and G β subunit association by using either HRP-linked FK2 monoclonal antibody or anti-G β (Figure 18 middle panel and bottom panel respectively).

Immunobloting for endogenous ubiquitin (Figure 18 middle right panel) revealed a large smear co-immunoprecipitating with the Flag- β_2AR when VCP was knocked down compared to the control siRNA condition, indicating that there is an increased ubiquitination of proteins associated with the receptor as a result of VCP knockdown. On the other hand, blotting for G β subunits (Figure 18 bottom right panel) again revealed a decreased interaction of Flag- β_2AR and endogenous G β under VCP knockdown. These results demonstrate that the increased amounts of Flag- β_2AR as a result of VCP knockdown do not necessarily associate with G β (or these complexes are less stable) and are increasingly associated with ubiquitinated proteins. These results are also consistent with previous results obtained with the proteasome inhibitor (Figure 13b). In preliminary BRET experiments, we also showed that knockdown of VCP reduced the interaction between G $\beta\gamma$ and the β_2AR as well

(Darlaine Pétrin, unpublished- data not shown), confirming our immunoprecipitation experiments.

Effect of VCP knockdown on cell surface expression of Flag-β₂AR

As seen with the proteasome inhibitor, levels of β_2AR that underwent complex glycosylation in the Golgi (represented by the 70 kDa band in Figure 18 marked with **) were also increased with VCP knockdown, suggesting that reducing VCP levels in HEK 293 enhances ER export and maturation of the receptor. To further test this idea, we performed confocal immunofluorescence experiments on permeabilized Flag- β_2AR stable cells transfected with VCP or control siRNA (Figure 19) in order to detect the location of Flag- β_2AR . Cell surface fluorescence was observed in both cases indicating that the trafficking itinerary of Flag- β_2AR was unaltered as a result of VCP knockdown. Consistent with western blot analysis, confocal experiments demonstrate that VCP knockdown leads to increased amounts of mature Flag- β_2AR at the plasma membrane. These results are also consistent with proteasome inhibition experiments performed earlier (Figure 14), once again demonstrating a critical role for VCP in ERAD-mediated processing of β_2AR in HEK 293 cells. We next decided to test the functionality of β_2AR under VCP knockdown.

Effect of VCP knockdown on intracellular signalling of β₂AR

To test the functionality of β_2AR following VCP knockdown, we tested the ability of endogenous β_2AR to stimulate adenylyl cyclase by again measuring cAMP production as a response to isoproterenol treatment in HEK 293 cells. HEK 293 cells were

transfected with either VCP or control siRNA, 72 hours later the ability of these cells to activate adenylyl cyclase was determined using the Tr-FRET assay (Figure 20a) as indicated earlier. The dose-response curves generated in response to isoproterenol were superimposable between VCP or control siRNA-transfected HEK 293, demonstrating that the VCP knockdown did not enhance the ability of the receptors to signal via the adenylyl cyclase pathway. Moreover we included a control condition in which HEK 293 were not transfected to demonstrate that transfection itself did not alter the ability of these cells to respond to isoproterenol and stimulate cAMP production. These results were also confirmed using a cAMP-sensitive BRET-based sensor, EPAC, in native HEK293 cells [190] (data not shown). We again decided to test the ERK1/2 MAP kinase pathway. Here, HEK 293 cells transfected with either control or VCP siRNA were followed by stimulation of endogenous β_2AR with isoproterenol or ascorbic acid for 5 minutes. Cell lysates were then subjected to western blot analysis (Figure 20b). Interestingly, basal levels of pERK1/2 were enhanced when VCP was knocked down as opposed to the control siRNA. Moreover, pERK1/2 response to stimulation by isoproterenol was enhanced when VCP was knocked down, although levels of total ERK were not altered, suggesting that VCP knockdown enhances ERK1/2 activation in HEK 293 cells via β_2 AR.

We also included a further control condition in which HEK 293 cells stably expressing HA-FP receptor were transfected control or VCP siRNA and stimulated with PGF2 α (Figure 20c). We observed the same pERK1/2 response in both conditions demonstrating that the VCP does not play a role in FP-mediated ERK1/2 regulation. This data is consistent with the idea that no general changes in ERK1/2 activatability

were caused by knockdown of VCP (or possibly treatment with MG-132), suggesting again that our results were receptor-specific. Overall this data indicates that VCP knockdown leads to increased expression of functional β_2AR which also enhances the signalling outcomes of β_2AR via MAPK while leaving signalling via adenylyl cyclase essentially unaltered.

RNF5 and gp78 interact with $\beta_2 AR$

In order to further elucidate the mechanism by which $\beta_2 AR$ is targeted to ERAD, we decided to confirm $\beta_2 AR$ interaction with several E3 ligases, resident in the ER, identified earlier in our MS screen (Table 3). gp78 E3 ligase have been previously shown to directly interact with VCP [146], therefore is probably involved in polyubiquitination of $\beta_2 AR$ in ER membranes. Interestingly another E3 ligase, RNF5, also identified in our MS screen which also ubiquitinates ERAD substrates [145, 151, 181]. To confirm their interactions with β_2AR , we performed coimmunoprecipitation or streptavidin bead purification experiments. A gp78-Flag construct was overexpressed in the TAP- β_2 AR stable cell line and Flag immunoprecipitation was performed followed by western blot analysis (Figure 21a). Immunoblotting with anti-HA demonstrated a specific band of approximatly 75 kDa corresponding to the TAP- β_2 AR co-immunoprecipitated with gp78-Flag (Figure 21 a left top panel). Similar results were obtained by performing the reverse experiment in which the same conditions were used but streptavidin bead purification was performed and gp78-Flag was immunoblotted using anti-Flag antibodies (data not shown). Also,

an RNF5-Flag construct was overexpressed in TAP- β_2 AR stable cells and streptavidin purification was performed, anti-RNF5 antibodies were used to show co-purification of endogenous RNF5 (36kDa) as well as RNF5-Flag with TAP- β_2 AR (Figure 21b lower panel). These results confirmed gp78 and RNF5, E3 ligases for ERAD as interacting partners of β_2 AR in HEK 293 cells, moreover these results validated our MS analysis of the Flag and TAP pulldowns.

DISCUSSION

Through the years, our understanding of GPCR signal transduction mechanisms has evolved from a simplistic model involving transient protein-protein interactions between receptors, effectors and G proteins to a model in which these signalling components form stable complexes with each other in order to mediate specific signalling outcomes. The formation of these "signalosomes" is particularly evident for prototypical receptor β_2AR which is implicated in treatments of various clinical conditions including heart failure. Previously published work from our laboratory demonstrated that $G\beta\gamma$ subunits and the ultimate effector adenylyl cyclase interact with the receptor at the level of ER [74, 157]. Unfortunately few studies focus on the events that occur during biosynthesis of GPCRs and factors mediating their assembly into singling complexes are unknown. Therefore, identification of novel interacting proteins of β_2AR within the ER might give us clues about how these signalling complexes are assembled and trafficked.

Section 1: Novel proteomic approach to identify interacting partners of $\beta_2 AR$

In order to identify novel interacting partners of β_2AR in the ER we developed and validated a gel-free proteomic purification strategy which makes possible the analysis of the β_2AR interactome. Proteomic analysis consists of biochemical purification of protein of interest coupled to MS analysis of the final eluate, resulting in identification of interacting proteins of this protein. The development of proteomic approach involved several stages, first being the selection of the purification approach itself.

In order to purify GPCRs and their interacting partners from their native milieu, the purification of the receptor should be performed from intact cells. Tandem affinity purification and FLAG affinity purification allow purification of GPCRs from cell lysates [90, 99-103, 176]. Both methods have distinct advantages. FLAG affinity purification involves fusion of relatively small epitope tag compared to the TAP thus reducing the possibility of the tag interfering with proper folding and function of the fused receptor. Moreover FLAG purification is based on one step affinity purification allowing for less stable interacting proteins to remain complexed with the receptor during the procedure [191]. TAP on the other hand consists of two-step affinity purification which permits an efficient removal of contaminants and identification of high-purity interacting partners [192]. Based on these advantages related to each technique we decided to develop both purification strategies for human $\beta_2 AR$. In order to perform each biochemical purification we fused either TAP or FLAG tags to the human $\beta_2 AR$ and generated two stable HEK 293 cell lines expressing either one of the receptors. The selection of the cells used for purification procedures determines the interacting proteins that are identified, in our study we chose to work with HEK 293 cells since these cells have been extensively used to study $\beta_2 AR$ signal transduction and trafficking, as well expressed proteins can be easily produced in quantities necessary for proteomic analysis. The receptors were expressed at physiological levels $(0.156 \pm 0.039 \text{ pmol/g})$ in stable TAP- β_2 AR and at supraphysiological levels $(3.470 \pm$ 0.519 pmol/mg) in the Flag- β_2 AR cells. We confirmed their appropriate trafficking to the plasma membrane and their ability to adequately respond to isoproterenol

treatment by detection of activated CRE-luc gene and phosphorylated ERK1/2 MAPK in stably expressing HEK 293 cells (Figure 4).

The second stage of proteomic purification development involves cell lysis, solubilization of the membrane bound receptors using appropriate detergents and performing small scale purifications. We were able to select detergents suitable for both purification procedures while retaining the functional properties of the solubilized receptor (Figure 5) and by performing small scale purifications we co-purified heterotrimeric G proteins with the tagged receptors (Figure 6). This co-purification gave us confidence that during both purification strategies we are able to retain functionally relevant interactants of β_2 AR [65].

The third stage in proteomic purification strategy development involves trypsinization of the sample and detergent removal prior to submit the sample for MS analysis. This procedure was previously done by subjecting the purified GPCRs to SDS-PAGE analysis and performing in gel-digestion and there are several examples where this technique was successfully used and resulted in identification of novel interacting partners [102, 103]. However such studies often fail to detect known interacting partners of the bait GPCR in their MS screens, thus failing to demonstrate that functional receptors have been purified along with their partners. In fact, for GPCRs, the only successful TAP study followed by MS analysis to date was performed using human melatonin receptors [176]. This group was able to successfully identify several isoforms of heterotrimeric G proteins well known to couple to melatonin receptors in their final MS analysis. However the list of identified proteins was limited and several other known interacting partners of the receptors

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were notably absent, suggesting that the proteomic approach could have been further optimized. The use of in-gel digestions of the final eluate prior to MS analysis is required to clear contaminants as well as to remove the detergent present in the final elution but this technique limits the identification of low abundance and membrane proteins as discussed in the introduction. As an alternative, in-solution digestion protocols allow for the trypsinization of proteins to occur directly in the final elution, therefore guarantees that all of the co-purified interacting proteins are digested. However, detergents present in the final elution must be removed since they interfere with subsequent MS analysis. The technique to remove the detergent from the final elution depends on specific properties of the detergent.

In the case of TAP, we used DDM as detergent of choice. This detergent has a low CMC and forms large micelles of an approximate weight of 70 kDa [193], thus techniques such as dialysis to remove this detergent cannot be applied. We decided to use a novel approach, FASP, to remove DDM and prepare the sample for MS. FASP was initially described by Wiśniewsk et al [109]. In their study, they were able to completely remove SDS from tissue and cellular lysates and digest the proteins retained in the filter unit. As a result, they were able to identify 2,000 proteins using 1-2 μ g of material in one single run, allowing for unprecedented proteome analysis. The elimination of the detergent occurs in the initial part of the protocol with urea washes that allow dissociation of detergent micelles [108]. The use of urea to remove SDS was based on their previous findings that demonstrated that detergents such as CHAPs, Triton-X-100 and SDS can be removed by urea which is thought to occur by increasing the CMC of detergents. Physical properties of these detergents are similar to DDM such as low CMC, non-ionic head group (for Triton-X-100) and the formation of large micelles [194]. Thus, we supposed that DDM can also be removed by urea during FASP. We used FASP to remove DDM from final elutions following large-scale TAP protocol and prepare the sample for MS analysis.

In the case of FLAG-based purifications, we were able to use OG as detergent of choice and its removal can be easily performed by ethyl-acetate extractions. Ethylacetate extraction allows for a quick and efficient removal of OG from in-solution digested samples based on solubility of this detergent in this organic solvent [110]. The water-saturated ethyl acetate extraction offers a significant advantage since it limits the handling of the sample during the detergent removal procedure as compared to FASP required in the TAP procedure.

We identified 97 interacting partners with TAP and 177 with FLAG-based purification schemes (common interactors from two independent purifications). We did not detect detergent contamination during the MS analysis, thus demonstrating proper removal of the detergents by both techniques. Classification of proteins identified in both purification procedures showed a diverse pattern of interactors (Figure 8 and 11), a similar pattern was observed in other proteomic studies of GPCRs such as for melatonin receptor [176] and is in line with recent studies indicating that GPCRs interact with various partners throughout their life cycles starting with biosynthesis in the endoplasmic reticulum (ER), maturation in the Golgi, and transport towards the cell surface. These interactors can modulate receptor signal transduction and internalization [195-198]. Comparing interactors identified by TAP and FLAG we detected a similar pattern of interactors originating from the same subcellular compartments and having the same intracellular functions, demonstrating that both purifications are able to detect similar β_2AR interactors. For our purposes we were interested in proteins located to the ER and these proteins represented 25% of total proteins identified following TAP and 14% by FLAG. In order to limit the number of interactors and detect specific interactors of β_2AR only at the level of ER, it would be beneficial to perform these proteomic approaches with dominant negative Sar1 and Rab proteins that regulate the β_2AR anterograde traffic in order to trap the receptor signalling complex in the ER. This would allow us to take proteomic snapshots of the receptor interactores in different subcellular compartments and at different points in their lifecycles.

In addition in our MS analysis we were able to detect several known interacting partners of β_2AR such as adenylyl cyclase, NHERF-2, in addition to heterotrimeric G proteins (Tables 1,2) giving us confidence that our purification procedures worked and are superior to previously reported proteomic screens of GPCRs, where known interactors were often missed. However, several other known interactions of β_2AR were not identified. Some interactions might be considered to be transient or agonist-stimulated which might underrepresent them in our screen since we performed our purification procedures on untreated cells. Interestingly, we were able to retain additional known interactors of β_2AR as a result of FLAG purification strategy as opposed to TAP and this can be explained by the possible loss of material during the FASP protocol as opposed to more simple ethyl acetate extractions. Moreover, we were able to identify the bait protein with almost the same coverage and amounts of peptides, for TAP peptides 9.5 and 24.4% percentage coverage and for

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FLAG 10 peptides and 19.5% coverage (Table 1 and Table 2), indicating that this highly hydrophobic protein was digested in a similar manner during the two purification methods since both of these methods employed in-solution digestion.

Overall, we were able to successfully purify β_2AR and identify known as well as novel interacting partners with the use of two proteomic methods. More importantly we were able for the first time to replace use of in-gel digestion protocols with insolution digestion protocols to study a GPCR proteome. Both methods allowed purification of the membrane proteins (such as adenylyl cyclase and β_2AR) as well as peripherally associated proteins (such as heterotrimeric G proteins) demonstrating that these methods are valuable in identify interacting partners of functional β_2AR under native conditions. Moreover, the purification methods described here can be used to study other GPCRs and their interacting networks. For example, several orphan GPCRs have unknown function therefore these methods can be used to characterize their signalling networks and shed light on their physiological functions. Ultimately uncovering the full set of GPCR-interacting proteins for receptors of clinical interest may provide novel therapeutic targets.

Section 2: Role of ERAD in regulation of β_2AR traffic and signalling complex assembly

ER-associated degradation (ERAD) is a process in which wild type, mutated or misassembled proteins such as GPCRs are degraded from ER. ERAD involves several steps including substrate recognition within the ER, targeting for degradation, retrotranslocation to the cytosol and degradation via ubiquitin-proteasome system (UPS) [1]. Our proteomic strategies (described in section 1) allowed us to identify several novel ER resident proteins as β_2AR interactors as a result of TAP and FLAG purifications. Careful analysis of these interactors indicated that several of them were implicated in each step of ERAD (Table 3). Interestingly, none of these factors have been previously reported to interact with β_2AR , leading us to hypothesize that the ERAD system can regulate the levels of this GPCR.

The functional presence of receptors and their associated signalling machinery at the plasma membrane is one key step for activation of their signal transduction mechanisms. Recently, the stability of $\beta_2 AR$ at the plasma membrane has been demonstrated to be regulated by the UPS system. This regulation is distinct from the ERAD pathway described above and has been extensively studied. It helps control receptor down-regulation from the cell surface as a result of ligand stimulation. Once the receptor is simulated by agonist, the receptor is phosphorylated and this allows for the recruitment of Nedd4, an E3 ligase, to the receptor in a complex with β -arrestin2 [35]. The ubiquitination of the receptor is then necessary to target the receptor for lysosomal degradation via a largely unknown mechanism, and it should be noted that the degradation of activated $\beta_2 AR$ occurs by lysosomes rather than the proteasome [177]. In contrast to the ERAD pathway, in which ER-resident E3 ligases ubiquitinate their substrates within the ER compartment and target them for degradation by the cytosolic proteasome. Thus, GPCRs that are degraded by ERAD within the ER have not been stimulated, since they never reach the plasma membrane in the first place, as a result this degradation occurs constitutively as part of an assembly or quality control function.

To confirm that steady-state levels of $\beta_2 AR$ are degraded by ERAD, we demonstrated that unstimulated $\beta_2 AR$ turn-over is indeed regulated by proteasomal degradation, rather than by lysosomal degradation in our stably expressing cells (Figure 12a). This result was not due to the ineffective treatments since HA-FP receptor was regulated by the lysosomal degradation under basal conditions (Figure 12b). Interestingly this receptor was not regulated by proteasomal degradation demonstrating that overexpression of a GPCR in HEK 293 cells does not overwhelm the capacity of the cells to process newly synthesized GPCRs leading to degradation by the proteasome. These results also indicate that some GPCRs are more prone to premature degradation than others. This selectivity may lay in intrinsic ability of some GPCRs to acquire their proper three dimensional structures within the ER. This was proposed for the δ -opioid receptor, luteinizing hormone and thyrotropin receptors since the irreversible misfolding and/or limitation in the folding kinetics of these receptors prolong their ER residence times and thus triggered their recognition as being aberrant, initiating their degradation [121, 125, 128]. Thus we may suppose that β_2 AR can be also inefficiently folded or more prone to misfolding in the ER and subject to premature degradation by ERAD. Alternatively, the association of the $\beta_2 AR$ or other GPCRs with the ERAD system might play some role in assembling specific signalosomes, sampling for the presence of key interactors. This may have implications for the mechanistic basis of biased signalling.

The role of cytoplasmic proteasome in degradation of newly synthesized $\beta_2 AR$ was confirmed with proteasome inhibitors, MG-132 and epoxomicin, which lead to accumulation of immature forms of the receptor in the cytoplasm. (Figure 13b, c). The immature form of the receptor represents the receptor form that did not acquire complex glycosylation pattern which is added by enzymes present in the Golgi [185]. The presence of this form of the receptor in the cytosol upon proteasome inhibition indicates an extraction of the immature from of the receptor from the ER membrane to the cytosol prior to its degradation, suggesting that newly-synthesized proteins are more prone for degradation. Similar results were obtained in studies using δ -opioid receptors and thyrotropin-releasing hormone receptors, where MG-132 treatment lead to receptor accumulation in the cytosol [125, 128]. In both cases, the receptors were present in unglycosylated and polyubiquitinated forms in the cytosol as a result of proteasomal inhibition. Polyubiquitination is a common modification that marks substrates for proteasomal degradation during ERAD and often these substrates are deglycosylated prior to degradation by the proteasome [155]. In our study, the cytosolic form of the receptor was in an unglycosylated form and co-purified with ubiquitin (Figure 13b), although we cannot conclude the ubiquitination status of the receptor due to constraints of our experimental design. The finding that full $\beta_2 AR$ polypeptide accumulates in the cytosol upon proteasome inhibition also demonstrates that the receptor does not get cleaved in the membrane and the function of the proteasome is not obligatory for retrotranslocation of β_2AR from the ER to the cytoplasm as it was proposed for some proteins to occur [152].

Proteasome inhibition also lead to an increased presence of $\beta_2 AR$ in membranes which also co-precipitated with ubiquitin, although once again we cannot conclude that the receptors were polyubiquitinated, this is a common modification that has been described to be crucial for GPCRs that are substrates of ERAD such as V2 vasopressin receptor, δ -opioid receptor, rhodposin and thyroptropin receptors [125, 126, 128, 199], thus we may suggest that this modification likely occurs for $\beta_2 AR$. Interestingly, the amount of $\beta_2 AR$ retained in the membranes that acquired complex glycosylation in the Golgi was also increased upon proteasomal inhibition (Figure 13b), similar to results obtained with δ -opioid receptors, rhodopsin and thyrotropin receptors [125, 126, 128], indicating that a proportion of newly synthesized receptors upon proteasome inhibition can exit ER and reach Golgi apparatus where they acquire their complex glycosylation pattern. Our results are different from results recently obtained by Xiao et al., where proteasomal inhibition did not lead to mature $\beta_2 AR$ accumulation, rather proteasomal inhibition lead to immature receptor stabilization in the ER compartment detected by immunofluoresent experiments in which the receptor colocalized with calreticulum an ER staining marker upon MG-132 treatments [177]. The discrepancy between our results can be explained by the different treatments times with MG-132, in their assay MG-132 treatments were performed for 24 hours in serum free media opposed to 5 hour treatments that we performed. Increased treatment times will further inhibit ERAD pathway leading to accumulation of unprocessed proteins within the cell. This may trigger the unfolded protein response which in turn triggers increased production of proteins related to ERAD and may trigger apoptosis

[200], ultimately this can change the processing of proteins within the cell and may affect levels of proteins necessary for proper trafficking of the receptor.

We further demonstrated that receptors in the membrane fraction escaping ERAD were able to retain their normal function by their increased ability to bind radioactive ligand suggesting that these receptors were not misfolded. However we cannot attest that all of the receptors escaping the ERAD are correctly folded since the radioligand binding experiment was performed on membrane preparations of stably expressing Flag- β_2 AR cells. As a result we detected an increased specific binding upon proteasome inhibition over control conditions but our assay was not designed to detect the total number of receptors (folded or misfolded) present in the membrane. In order to perform these assays we need to label metabolically newly synthesized receptors as it is done in pulse-chase experiments, and then treat cells with proteasomal inhibitors and perform the radioligand binding assays. In this paradigm, we will be able to deduce the proportion of newly synthesized receptors that can bind the radioligand properly and thus are properly folded versus the ones that cannot and are thus misfolded. Nevertheless, these findings suggest that some receptors that are degraded by the proteasome are not irreversibly misfolded but possibly represent a folding intermediate due to kinetic limitation in its folding similar to what has been proposed for δ -opioid receptors [125]. Thus, allowing more time for $\beta_2 AR$ to fold within the ER, as it is the case during proteasome inhibition, allows the receptor to acquire its proper three dimensional structures within the ER and promotes its ER exit and traffic to the Golgi apparatus.

Since some proportion of receptors rescued from proteasomal degradation can fold correctly within the ER, we supposed that they can associate with their interacting partners. We decided to test the ability of these receptor to associate to $G\beta\gamma$ subunits since this interaction has been shown to occur at the level of ER [74]. We detected a decreased co-purification of $G\beta\gamma$ subunits upon proteasomal inhibition with the receptor (Figure 13b), although the proper folding of the receptors was increased. This data suggests that normally, receptors that are uncomplexed with $G\beta\gamma$ subunits are targeted to degradation via ERAD. We should confirm this finding by demonstrating a decreased interaction between receptor and $G\beta\gamma$ with another assay such as BRET in which we can quantify the interaction between the tagged receptor and tagged $G\beta$ or γ subunits under proteasome inhibition.

In addition using cell surface confocal immunofluorescence experiments we confirmed that receptors that are rescued from ERAD can properly be trafficked to the plasma membrane (Figure 14), and we decided to probe the signalling pathways that are activated by these cell surface β_2ARs in HEK 293 cells. We detected the same pattern of cAMP production as a result of β_2AR activation in both control and MG-132 treated cells, demonstrating that the increased receptor levels are unable to activate cAMP pathway, thus representing a decreased ability of these β_2ARs to stimulate adenylyl cyclase (Figure 15a). This effect could be explained by the decreased ability of the ERAD rescued receptors to associate with adenylyl cyclase. β_2AR and adenylyl cyclase have been shown to interact at the level of ER and our recent work suggests that $G\beta\gamma$ subunits are important in the assembly of receptor/effector complexes [74, 157, 201] and their role in orchestrating this

assembly in the ER is only starting to become appreciated reviewed in [201]. Thus the lack of receptor interaction with GBy subunits may also entail a decreased association (complex formation) of the receptor with adenylyl cyclase at the level of ER. Thus receptors rescued from ERAD may be unassembled or differently assembled signalling complexes, i.e. receptors that lack the interaction with effector and G proteins, resulting in increased cell surface receptors that are unable to activate the cAMP/PKA pathway. The idea of premature degradation of unassembled signalling GPCR complexes is supported with a study performed on luteinizing hormone receptor, where an increased level of monomeric receptors that gained hormonal binding capability were detected as a result of proteasome inhibition [202], although this receptor has been previously shown to form dimers within the ER [203]. Therefore, ERAD targets functional GPCRs that are either misfolded and/or misassembled with other proteins within the ER. In our case it seems that the receptors targeted to degradation is uncomplexed with adenylyl cyclase and $G\beta\gamma$, it would be crucial to further assess and confirm the effect of proteasome inhibition on $\beta_2 AR$ interactions with adenylyl cyclase and $G\beta\gamma$ with assays such as BRET and immunoprecipitations (in the case adenylyl cyclase) to determine if this notion is true.

On the other hand, we were able to enhance activation of the ERK1/2 pathway by β_2 ARs in HEK 293 pre-treated with proteasome inhibitor (Figure 15b), suggesting that rescued receptors are able to activate this pathway. However, results pertaining to this signalling pathway activated by rescued receptors should be carefully analyzed since as mentioned before MG-132 inhibits the endocytosis of the receptor and this may also explain the enhanced signalling by ERK1/2. Moreover ERK1/2 can be regulated by proteasome degradation, in a study performed by Yang et al., and MG-132 treatment promoted a sustained ERK1/2 response [204]. Thus this regulation can also explain the increased ERK1/2 response in our assay. In order to eliminate the confounding effects related to the MG-132 treatments, and to study the mechanism by which the unassembled complex is targeted to degradation, we decided to test the association of β_2 AR with VCP, which we confirmed with co-immunoprecipitation assay (Figure 16).

VCP's role in substrate retrotranslocation from the ER to the cytoplasm has been reported for multiple substrates of ERAD including one GPCR, vasopressin receptor [146, 182, 187]. Studies using the V2 vasopressin receptor only demonstrated immunoprecipitation of the receptor with VCP and did not study the effect of this protein on the receptor level [199], we on the other hand over expressed and knockdown this protein in order to confirm its role in retrotranslocation of the receptor. In addition to its role in ERAD, VCP has been implicated in numerous other cellular functions including membrane fusion [205, 206], nuclear trafficking [207] and cell proliferation at the level of both cell division and apoptosis [208, 209]. Ultimately the role of VCP depends on its association with adaptor proteins. VCP's role in ERAD is mediated by its association with the Ufd1-Npl4 heterodimer [210]. The complex Ufd1-VCP-Npl4 is thought to bind first to polypeptide backbone and then to the polyubiquitin assembled on the ERAD substrates [155]. In our MS screen, we identified all three factors (Ufd1-VCP-Npl4) leading us to believe that VCP interactions with $\beta_2 AR$ are related to the ERAD. Hydrolysis of ATP by VCP provides the necessary energy to complete retrotranslocation of ERAD substrates [140]. VCP also chaperones polyubiquitinated proteins to the proteasome for degradation by direct association with the proteasome [154]. Thus knockdown of VCP should arrest the retrotranslocation of β_2AR from ER and the levels of rescued β_2AR should be similar to the ones obtained with proteasome inhibitor.

In our stable TAP- β_2 AR cells, knockdown of VCP mimicked the recovery that we observed with MG-132 treatments suggesting that VCP plays a critical role in turnover of the receptor (Figure 17a). HA-FP receptor was unaffected by this treatment demonstrating that VCP does not affect the expression level of this receptor (Figure 17b), similar to results obtained with proteasome inhibitor once again indicating that some GPCRs such as β_2 AR are more prone for ERAD. Overexpression of VCP with proteasomal inhibitor treatment, revealed an increased form of the cytosolic immature receptor form compared when the cells were exposed to the proteasome inhibitor alone, demonstrating that retrotranslocation of β_2 AR is performed by VCP protein (Figure 17c).

Knockdown of VCP also caused an accumulation of unglycosylated and complex glycosylated forms of the receptors bound to membranes, and these forms of receptors increasingly co-immunoprecipitated with ubiquitin (Figure 18). These results are in line with the fact that ERAD substrates are first polyubiquitinated within the ER prior to their association with VCP since the polyubiquitin moiety provides a handle for the VCP complex to initiate an ATP-dependent extraction [154]. β_2 AR under VCP knockdown, as with proteasome inhibition, acquired complex Golgi glycosylation (Figure 18) and were delivered to the plasma membrane as detected by immunofluorescence experiments (Figure 19). However, we were not using clonally selected Flag- β_2 AR cells, thus the expression level of receptor in each cell can vary. The caveat of immunofluorescence experiments is that it focuses on one cell, thus staining obtained as a result of treatment can be confounded by the variation in expression levels of each cell, therefore other techniques such as cell surface biotinylation followed by an immunoprecipitation of the receptor should be performed to confirm the increased presence of the receptor on the plasma membrane. Despite this limitation we constantly detected increased levels of β_2 AR at the plasma membrane in several cells and in two independent experimental set ups (one with proteasome inhibitor (Figure 14) and another with siRNA treatments (Figure 19) giving us confidence that rescued receptors from ERAD can travel to the plasma membrane.

In addition we demonstrated a decreased association of $G\beta\gamma$ with β_2AR as a result of VCP knockdown (Figure 18). Once again confirming the fact that β_2AR destined to be degraded by the ERAD do not interact with $G\beta\gamma$, suggesting an unassembled or perhaps differentially assembled signalling complex. These results provide additional evidence that β_2AR is a substrate of ERAD occurring through the action of VCP and VCP is necessary for the retrotranslocation of uncomplexed β_2AR -G $\beta\gamma$ to the cytosol for proteasome mediated degradation.

The signalling properties of β_2AR under VCP knockdown were exactly the same as previously described with the proteasome inhibitor studies (compare Figure 15 and 20). These results further confirm the idea that receptors that are rescued from ERAD are unable to activate the cAMP/PKA pathway most probably due to lack of its association with G $\beta\gamma$ subunits. The activation of pERK response was enhanced with

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rescued β_2AR and this observation was confirmed with VCP siRNA, in addition we included another control in which HEK 293 cells stably expressing HA-FP were also treated with VCP siRNA and the ability of HA-FP receptor to activate pERK was tested. This was an important control demonstrating that the stability of ERK1/2 was unaffected by siRNA treatments, unlike with MG-132, and the result that we observe is specifically due to the effect mediated via β_2ARs .

In order to identify which signal transduction pathway of $\beta_2 AR$ is responsible for the ERK activation, we need to study the signal transduction pathways that activate ERK by β_2 AR in HEK 293 cells. The activation of ERK responses in HEK 293 cells by $\beta_2 AR$ involves several pathways which can be divided in to two broad categories, G protein-dependent and post G protein, β -arrestin-dependent pathways [211]. G protein-dependent activation can occur through the receptor coupling with either Gas or Gai [26, 28]. It has been reported that when using a balanced (unbiased) agonist of β AR such as isoproterenol both pathways are activated [211]. We think that the G protein-dependent activation of ERK response is less likely since we detected decreased association of rescued receptors with $G\beta\gamma$. Previous studies demonstrated that recruitment of the Gas subunit to form the heterotrimeric G protein occurs postrecruitment of the $G\beta\gamma$ subunits to the signalling complex [212], thus we can suppose that ERAD rescued receptors association with Ga subunit is also decreased. We believe that the enhanced ERK response that we observed is likely to occur through βarrestin dependent activation, since this activation may not require the heterotrimeric G proteins complexed to the receptor, if the receptor can get to the cell surface and recruit β -arrestin. This type of activation was demonstrated by $\beta_2 AR$ mutant that was incapable of G protein activation but can activate β -arrestin-dependent ERK [211]. The mutant resembles the ERAD-rescued receptors since they cannot activate G protein dependent signalling (at least via adenylyl cyclase) and do not associate with Gβy, however they can activate the ERK response. This idea should be further tested with additional experiments such as a performing live-cell immunofluorescence experiments under protreasonal or VCP knockdown to follow the recruitment of β arrestin to the receptor, as well we could perform a time-course experiments of MAPK assays, since the activation pattern of G protein-dependent ERK1/2 and post G protein ERK1/2 activation is different. It has been observed that ERK1/2 response generated by G protein-dependent signalling is rapid and declines quickly within the first 5 minutes and β -arrestin dependent activation is delayed and is sustained up to 20 minutes [211]. Performing a time course will thus indicate the nature of pERK response activated by the rescued β_2ARs from ERAD. We should also test the possibility, however unlikely due to decreased association with $G\beta\gamma$, that the rescued ERAD receptors can associated to the $G\alpha$ is subunit and can activate ERK this way, this should be determined experimentally by including a Gai inhibitor (pertussis toxin) in addition to proteasome inhibitor while performing the MAPK assays, in this set up the G protein-dependent activation should be abolished completely. Nevertheless our data demonstrated that receptors that are targeted to the ERAD are able to have a biased signalling profile, thus can selectively activate one signal transduction pathway other another. This could be due to either differential signalling complex assembly or to the reduced effector or G protein association with the receptor.

VCP has been described to acts as a segregase, since it can recognize the ubiquitinated substrates other than ones that are not ubiquitinated within the ER [213]. It is interesting that some β_2AR forms, such as the ones that can signal through ERK1/2 pathway are selectively degraded by ERAD. E3 ligases recognize and target specific substrates for ERAD and thus can be thought to mediate substrate recognition during ERAD [147]. We identified two E3 ligases as interactants of β_2AR , gp78 and RNF5 and confirmed their interaction with β_2AR (Figure 21). Although we tried to determine their effects on the expression levels of the receptor and on the signalling properties of the receptor our results were inconsistent and inconclusive. This may be due to the cooperation between these E3 ligases, as it was demonstrated to occur for degradation of CFTR [145]. In addition several ERAD substrates require the activity of several E3 ligases to target them for degradation [147]. This may hint that redundancy and cooperation between E3 ligases making their study more difficult.

An intriguing question remains how does the cell recognize and target β_2AR uncomplexed with G $\beta\gamma$ for degradation via ERAD and not the receptors that are complexed to the G $\beta\gamma$ subunits. The answer may lay in the structural flexibility of the receptor. This flexibility is thought to be necessary for the receptor to induce different conformations and activate several signalling pathways [45]. However, this flexibility may make β_2AR more prone to misfold in ER and thus target it for premature degradation as discussed previously. Interestingly several wild type GPCRs such as gondotropin receptor, vasopressin receptor, δ -opioid receptor functional expression at the plasma membrane can be enhanced in the presence of pharmacochaperones [129, 130, 132]. These cell permeable ligands, specifically bind to intracellularly retained GPCRs and are thought to stabilize their flexible structure and thus promote their proper folding, as a result increased amount of properly folded and functional receptors are able to escape from the ERAD and traffic to the plasma membrane. We may thus think that formation of protein interactions (such as with $G\beta\gamma$, adenylyl cyclase and β_2AR monomer in dimerization) within the ER may also serve a stabilizing function, much like the use of pharmacochaperones, by limiting the flexibility within the $\beta_2 AR$ structure and thus promoting its proper folding within the ER. This idea has been proposed in studies focusing on homo and heterodimerization of GPCR within the ER [214]. Indeed, several studies have indicated that the ER exit of some GPCRs and subsequent traffic to the cell surface requires dimerization in the ER [215]. In fact, β_2 AR homodimerization within the ER is a required step for its traffic to cell surface supporting the idea that receptors interactions within the ER promotes receptors proper folding and allows its ER exit [50]. Moreover, other studies indicated that subunit assembly/maturation efficiency in the ER only ranges between 20 and 40% for a variety of oligometric ion channels [216-218], suggesting that ERAD may have a role in the oligomeric channel assembly. Thus we can propose that the role of ERAD is not only to control the number of functional wild type $\beta_2 AR$ but to have a significant and previously uncharacterized role during receptor complex assembly.

Interestingly, the activation of ERK by β_2AR via the β -arrestin mediated mechanism has been shown to provide beneficial outcomes during heart failure [41], the same pathway that we think was activated by β_2ARs escaping ERAD. Thus, specifically inhibiting ERAD of β_2AR may represent a novel therapeutic avenue in this clinical situation. Although we demonstrated that VCP inhibition specifically inhibits ERAD of β_2AR , its implication in several other cellular functions and ability to associate to various other ERAD proteins makes this an unspecific drug target. We should further analyze which factors within the ER are able to specifically target this uncomplexed form of β_2AR to degradation. In order to do so, we should repeat our proteomic purification methods described in section 1 in conditions where ERAD of β_2AR is inhibited (such as under VCP knockdown) in hopes of identifying several other proteins related to substrate recognition during ERAD. The identification of specific factors regulating β_2AR ERAD may represent valuable drug targets that can be selectively inhibited using peptidomimetic or small molecules inhibitors during heart failure.

An issue related to our studies, is the use of HEK 293 cells throughout our experiments. Although HEK 293 cells express ubiquitous set of proteins they may lack specific β_2AR interacting partners found in specialized cells such as cardiomyocytes. Thus the assays described here should be performed in these cells in order to identify cell type specific interactors of β_2AR and demonstrate that ERAD has similar functions in controlling the levels of β_2AR and its signalling complex assembly.

SUMMARY

We successfully developed two novel proteomic approahes to study β_2AR interacting partners, as a result we identified several ERAD proteins as interactors of β_2AR . We confirmed three novel interactions of β_2AR with VCP, gp78 and RNF5,

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proteins related to different ERAD functions within cells. We further demonstrated that β_2AR is a substrate of ERAD in HEK 293 cells (summarized schematically in Figure 22). In addition, we demonstrated that β_2ARs released from ERAD are not associated to their signalling partner $G\beta\gamma$ and the receptors arriving at the plasma membrane have a biased signalling profile, such that they activate the pERK pathway as oppose to cAMP/PKA. Our results suggest a novel mechanism by which β_2AR signalling complex assembly can be monitored by ER qualiy control via ERAD and this novel regualtion can be used in future therapeutic strategies.

FIGURES AND TABLES



Figure 1: *Diverse signalling pathways regulated by* β_2AR . β_2AR can activate several signalling pathways following agonist stimulation. Activation of the receptor leads, in turn, to activation of both Gas and Gai which both regulate activity of adenylyl cyclase (AC) affecting the production of cAMP. cAMP activates protein kinase A (PKA) which can phosphorylate and regulate several other proteins including β_2AR . Other signalling pathways are activated by c-Src and by G $\beta\gamma$ subunits- examples include p38 MAPK and ERK1/2 respectively. Receptor desensitization involves receptor phosphorylation by GRKs, PKA and PKC, recruitment of β -arrestins and internalization into endosomes. This process also activates several other signalling pathways including ERK1/2. Endocytosed receptor is either recycled to the plasma membrane for another round of activation or ubiquitinated by Nedd4, targeting the receptor for lysosomal degradation.



Figure 2: Schematic representation of proteomic approach to identify novel interacting partners of GPCRs. 1) Receptor complexes are first isolated following cell lysis and solubilized with detergents. Detergent of choice should encapsulate the receptor and associated proteins within micelles. 2) To isolate specific interactors, biochemical purification of the receptor of interest is performed using several methods (see text for details). 3) The final elution containing co-purifying interacting proteins is then prepared for mass spectrometry analysis (MS). This is done by performing either in-gel or gel-free digestion. In-gel digestion involves resolving the final eluate by 1- or 2D SDS-PAGE, followed by gel excision and trypsinization. In-solution methods involve in-solution trypsinization and removal of detergent from resulting peptide mixtures. 4) The purified peptides are ready for mass spectrometry (MS) analysis such as liquid chromatography tandem mass spectrometry (LC-MS/MS).



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Figure 3: Overview of steps involved in ER-associated degradation (ERAD). **a**) ERAD begins with detection of aberrant proteins by several factors including cytosolic and intracellular chaperones, nucleotide exchange factors, calnexin and ER degradation enhancing α -mannosidase-like lectins (EDEMs). **b**) The selected protein is then targeted for ubiquitination by an E3 ligase which is resident of ER and the protein is delivered to the retrotranslocon. **c**) Retrotranlocation is mediated by Cdc48 (in humans VCP) which is often associated with other factors such as NPL4-UFD1. VCP can also associate with the proteasome directly. **d**) The retrotranslocating substrate can be further ubiquitinated with other E3 ligases. **e**) Degradation of the substrate in the cytosol occurs in the proteasome which may be associated with de-ubiquitylating or to de-glycosylation enzymes. Figure taken from [1].



Figure 4: *Functional expression of Flag-\beta_2AR and TAP-* β_2AR *in stably transfected HEK 293 cells.* **A)** Schematic representation of TAP- and Flag-tagged receptors. **B)** Plasma membrane localization of the Flag- β_2AR and TAP- β_2AR was monitored by confocal immunofluorescence microscopy on non-permeabilized cells. Receptors were detected using either polyclonal Anti-Flag or monoclonal anti-HA antibodies. **C)** Cre-Luc construct were transfected into either Flag- β_2AR or TAP- β_2AR stable cell lines, followed by 6 hour treatments with 1µM isoproterenol, 0.1 µM ascorbic acid (vehicle), or left untreated. Cells were lysed and luciferase activity was measured and normalized to the luciferase activity obtained in the untreated condition. **D)** Transfected HA- β_2AR cells, or stably expressing Flag- β_2AR and TAP- β_2AR HEK 293 cells were treated with 10 µM isoproterenol or 0.1 µM ascorbic acid (vehicle) for the indicated times. Levels of activated ERK1/2 were visualized by immunoblotting phospho-ERK normalized to total ERK levels. Data are representative of 3 independent experiments.



Figure 5: *TAP-\beta_2AR retain the ability to bind ligand after solubilization.* Crude membrane preparations with 0.5% DDM of **A**) native HEK 293 cells and **B**) TAP- β_2AR stably expressing HEK 293 cells were subjected to column filtration following ¹²⁵I-CYP binding described in *Material and Methods*. Data is presented from a single experiment performed in triplicate.



Figure 6: *Tandem Affinity Purification of* β_2AR *complexed with* $G\beta\gamma$. **A)** Schematic representation of the dual affinity purification protocol TAP. **B)** TAP- β_2AR stable HEK 293 cell lines were subjected to TAP and specific fractions throughout the TAP procedure were kept for western blot analysis. Lane 1: Input receptor, lane 2: supernatant post-streptavidin beads binding, lane 3: washes post-streptavidin beads binding, lane 4: elution from streptavidin beads, lane 5: supernatant post calmodulin beads binding, lane 6: washes post-calmodulin beads binding, lane 7: elution from calmodulin beads. **C)** HEK 293 cells or TAP- β_2AR stable cell line were transfected with either pcDNA3.1, $G\beta_1$ -Flag, $G\beta_2$ -Flag, $G\beta_4$ -Flag all co-expressed with GasEE and G γ 2-HA to reconstitute the heterotrimeric G protein. Following TAP, calmodulin elutions were separated by SDS-PAGE gel, anti-HA antibodies were used to detect receptor and anti-G β antibody was used to detect levels of endogenous or heterologously expressed G β subunits from heterotrimeric G proteins. Data representative of at least two independent experiments in each case.



Figure 7: *Schematic representation of FASP protocol.* 1. Final elution from calmodulin beads following scaled-up TAP protocol was applied on top of YM-10 microcon concentrator which contains a YM membrane that retains proteins above 10 kDa. 2. Following addition of 8M urea and subsequent centrifugation, the proteins from the calmodulin elution are dentaured and retained in the YM-10 microcon concentrator. In addition, the buffer containing DDM is exchanged. 3-4. Denatured proteins are then trypsinized overnight within the unit. 5. In order to eliminate non-specific binding of peptides to the YM membrane, 5M NaCl was added to the denatured peptides. 6. Peptides in 5M NaCl solution are collected following centrifugation of the unit. 7-8. Peptides were then applied on to 3M empore extraction columns which allow buffer exchange and result in peptides suitable for LC-MS/MS.


	TAP #1	TAP #2
Total Number	250	183
of Hits		
Common Hits	9	7

Figure 8: Analysis of interacting proteins of β_2AR identified by mass spectrometry following TAP. Desalted and detergent free peptides obtained following two independent TAP purifications were analyzed by liquid chromatography- tandem mass spectrometry (LC-MS/MS), and common set of proteins (hits) present in both purifications were identified. These interactors where then classified according to function or location using NCBI databases. Data is representative of two independent experiments.

Gene Symbol	Protein Identity	Number of Unique Peptides	Number of Total Peptides	Coverage (%)	n
Bait Protein					
ADRB2	β_2 -adrenergic receptor	9.5	29.5	24.4	2
Interacting Proteins					
NSF	N-ethylmaleimide- sensitive factor	10.5	14	17.3	2
GNAI3	G protein, alpha inhibiting activity polypeptide 3	3	3	10.7	1
UBB	Ubiquitin B	3	7	53.2	2
SLC9A3R2/NHERF2	Solute Carrier Family 9 (sodium/hydrogen exchanger), member 3 regulator 2	3	4	12.3	1

Table 1: Mass Spectrometry results identifies known interacting partners of β_2AR following *TAP protocol*. Percent coverage indicates the percent of total amino acid sequence covered by peptides identified through mass spectrometry. Number of unique peptides as well as number of total peptides are indicated as mean values. n represents the number of independent experiments during which a particular protein was identified.



Figure 9: *FLAG purification of* β_2AR *complexed with* $G\beta\gamma$. **A)** Schematic representation of FLAG purification. **B)** Flag- β_2AR cells or native HEK 293 cells were subjected to FLAG purification. Eluates were separated by SDS-PAGE, anti-Flag antibodies were used to detect receptor and anti-G β antibody was used to detect levels of endogenous or heterologously expressed G β subunits from heterotrimeric G proteins. * represents monomeric form of the receptor and ** represents oligomeric form of the receptor. Note that some G β sticks to the Flag beads in the control condition. Data representative of at least two independent experiments.



Figure 10: *Ethyl acetate extraction of octylglucoside detergent.* 1-2. Final elution of FLAG purification was incubated with trypsin overnight. 3-4. Water-saturated ethyl acetate solution was added to the trypsinized sample. The upper layer contained ethyl acetate with the detergent. The lower layer contained water soluble peptides. The detergent is thus removed by simple extraction. 5. In order to ensure that all of the octylglucoside contained in the ethyl acetate layer was removed, the final extraction was followed by centrifugal evaporation. 6. The final lyophilized peptides resuspended in a LC-MS/MS compatible buffer are ready for analysis.



Molecular Function



Gene Symbol	Protein Identity	Number of Unique Peptides	Number of Total Peptides	Coverage (%)	n
Bait Protein					
ADRB2	β_2 -adrenergic receptor	10	22	19.5	2
Interacting Proteins	· ·				
ADCY3	Adenylyl Cyclase	1	1	1.7	1
NSF	N-ethylmaleimide sensitive factor	10	15.5	16.3	2
GNB1	Guanine nucleotide- binding protein, beta 1 subunit	4	3	13.5	1
GNB2	Guanine nucleotide- binding protein, beta 2 subunit	1	1	1	1
GNB	Guanine nucleotide- binding protein, beta subunit (1-4)	3	2	6.2	1
GNAI3	Guanine nucleotide binding protein, alpha inhibiting	2	2	10.2	1
GNG12	G protein gamma 12 subunit	1	1	22.2	2
UBB	Ubiquitin B	1	1	21	1
SLC9A3R2/NHERF2	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	4	5	17.2	1

Table 2: Mass Spectrometry results identifies known interacting partners of $\beta_2 AR$ following FLAG protocol. Percent coverage indicates the percent of total amino acid sequence covered by peptides identified through mass spectrometry. Number of unique peptides as well as number of total peptides are indicated as mean values. n represents the number of independent experiments during which a particular protein was identified.

Gene	Protein Name	Unique	Total	Coverage	Ref.
Symbol		Peptides	Peptides	(%)	
AMFR	Autocrine Motility Factor	3.2	4	4.68	[219]
	(gp78)				
RNF5	Ring Finger Protein 5 (RNF5/RMA1)	1.33	3.33	11.03	[181]
VCP	Valosin-containing Protein (p97/VCP)	12	19.67	20.33	[220]
NPL4	Nuclear Protein Localization 4	3	3.33	6.9	[153]
UFD1	Ubiquitin Fusion Degradation 1	1	1	4.1	[153]
HSPA5	Heat Shock 70 kDa protein 5 (glucose regulated protein, 78 kDa)	17.17	33.5	33.5	[134]
HSPA1B	Heat Shock 70 kDa protein 1B	16.5	35.67	35.06	[134]
DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11 precursor	1.67	1.67	6.33	[135]
CANX	Calnexin	10.83	23.8	16.77	[137]
SEC61A1	Sec61 alpha 1 Subunit	2.4	2.8	7.78	[139]
PSMD2	Proteasome26S non- ATPase subunit 2	2	2	2.8	[124]
KIAA0368	ECM29	5.5	4	2.15	[180]

Table 3: Proteins involved in ER Quality Control and/or ER-associated degradation identified as β_2AR interactors as a result of several TAP and FLAG purifications followed by mass spectrometry analysis. Coverage represents the percent of the total amino acid sequence of a protein covered by unique peptides, n represents number of times that a particular protein was identified in independent purifications.



Figure 12: Selective effects of proteasome inhibition on β_2AR . Cells stably expressing TAP- β_2AR (A) or HA-FP (B) were treated with the protein synthesis inhibitor, cycloheximide (CHX) 5 µg/ml, alone or in conjunction with the proteasomal inhibitor, MG132 (10 µM), the lysosomal inhibitor, NH₄Cl (30 mM), or vehicle, DMSO (0.1%) for indicated times. Cells were lysed in a hypotonic lysis buffer containing DDM, the resulting lysates were then analyzed by western blot. Anti-HA antibodies were used to detect both receptors and GAPDH was used as loading control. Data representative of 3 independent experiments.



Figure 13: Proteasomal inhibition alters receptor trafficking and interactions. A) Flag- β_2 AR cells or native HEK 293 cells (NT) were treated for 5 hours with MG-132 in serum free media. Cells were lysed in hypotonic lysis buffer followed by a 100,000 X g centrifugation to separate membranes from cytosol. Membranes were solubilized overnight in solubilization buffer. Total lysates were assessed for fractionation efficiency immunobloting with anti-Na⁺/K⁺-ATPase and anti- β -tubulin. bv B) Flagimmunoprecipitations were performed on solubilized membranes as well as on the cytosolic fraction described in A) and the finale eluate was analyzed by western blot. Receptor was immunobloted with anti-Flag, endogenous ubiquitin was immunoblotted with HRP-linked FK2 monoclonal antibody and endogenous GB with anti-GB. C) TAP- β_2 AR or HA-FP stable cells were treated for 5 hours as in Figure 12 with the addition of epoxomicin 1 µM (another proteasomal inhibitor). The cells were then lysed in a hypotonic lysis buffer and the cytosol was separated from membranes by a 100,000 X g centrifugation. Anti-HA antibody was used to visualize both receptors and anti-β-tubulin was used to detect β -tubulin levels as a loading control. Data representative of two experiments. ** represents glycosylated mature receptor forms and * immature monomeric form of the receptor.



Figure 14: Rescued β_2AR from proteasomal degradation are able to correctly traffic to the plasma membrane. HEK 293 cells stably expressing Flag- β_2AR were treated with 10 µM MG-132 or DMSO for 5 hours. Flag- β_2AR localization was determined using anti- β_2AR antibodies. HEK 293 cells were used as negative control for non specific antibody binding. Data are representative of two independent experiments.



Figure 15: Differential effect of proteasomal inhibition on intracellular signalling pathways activated by β_2AR . A) Dose-response curve of cAMP generated in HEK 293 cells as a function of increasing concentrations of the βAR agonist, isoproterenol. cAMP levels were detected using the LANCE Ultra cAMP Kit on HEK 293 cells pre-treated with either 10 μ M MG-132 or 0.1% DMSO cells. Data are representative of 2 independent experiments. B) Western blot analysis of ERK1/2 activation by β_2AR in HEK 293 cells following proteasome inhibiton. HEK 293 cells pre-treated with either MG-132 or DMSO were stimulated with 1 μ M isoproterenol for 5 minutes. Cells were then lysed and western blot analysis was perfomed to detect levels of pERK and total ERK with anti-ERK/p44/42(T202/Y204) and anti-ERK-ct antibodies respectively. Data are representative of three independent experiments.



Figure 16: *Validation of VCP interaction with* β_2AR . Native HEK 293 cells or HEK 293 cells stably expressing Flag- β_2AR were transfected with VCP-myc. 48 hours later, cells were lysed in hypotonic lysis buffer. Membranes were collected as a result of 100,000 X g centrifugation and resuspended in solubilization buffer containing 0.5% DDM, on which immunoprecipitation was performed using Flag-agarose beads. Solubilized membrane preparations prior to immunoprecipitation (input) (**A**) and final eluates (**B**) were subjected to western blot analysis and probed with anti-myc and anti-Flag antibodies to detect levels of VCP-myc and Flag- β_2AR respectively. Data are representative of two experiments. # represents non-specific bands, *** represents oligomeric structures, ** complex glycosylated receptors, * monomers. Data represent two individual pulldown experiments.

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Figure 17: *Role of VCP in regulation of* β_2AR *levels in HEK 293 cells*. **A)** TAP- β_2AR or **B)** HA-FP stable cells were transfected with VCP-myc in conjunction with either 30 nM *VCP* or 30 nM of *control* siRNA. 72 hours later total cell lysates were collected and the levels of VCP were assessed using anti-myc antibody, anti-HA antibody was used to detect the levels of TAP- β_2AR or HA-FP and anti-GAPDH was used as a loading control. **C)** Flag- β_2AR stable cells were transfected with either VCP-myc or pcDNA3.1, the cells were then treated for 5 hours with either 10 μ M MG-132 or 0.1 % DMSO. Cell lysates was fractionated into cytosol and membranes by a 100,000 X *g* centrifugation. The cytosolic fraction was then probed with anti-myc to detect VCP levels, anti-Flag to detect Flag- β_2AR and β -tubulin antibody was used as loading control. Data are representative of at least three independent experiments in a, two for b and one for c. * represents immature form of the receptor.



Figure 18: *VCP knockdown alters receptor trafficking and interactions*. Flag- β_2 AR stable cell lines were transfected with 30 nM *control* siRNA or 30 nM *VCP* siRNA, 72 hours later the cells were lysed in a hypotonic solution followed by cellular fractionation with a 100 000 X g spin to separate membranes and cytosol. Membranes were solubilized in solubilization buffer. Flag immunoprecipitation was performed on solubilized membranes as well as on the cytosolic fractions obtained. Anti-Flag antibodies were used to detect the level of the receptor, anti-ubiquitin was used to detect the levels of endogenous ubiquitin and anti-G β was used to detect endogenous levels of G β . # represents non-specific bands, *** oligomeric structures, ** complex glycosylated receptor structures and * monomeric receptor. Data are representative of at least two independent experiments



Figure 19: *VCP knockdown does not alter* β_2AR *trafficking to the plasma membrane.* Stably expressing Flag- β_2AR cells were transfected with either VCP or control siRNA. 72 hours later, cells were prepared for confocal immunofluorescence. To visualize the location of Flag- β_2AR , anti- β_2AR antibodies were used. HEK 293 cells were used as negative control. Data are representative of multiple fields from a single experiment.



Figure 20. Differential effects of VCP knockdown on signalling properties of $\beta_2 AR$ in HEK 293 cells. A) Dose-response curve of cAMP generated in HEK 293 cells as a function of increasing concentration of BAR agonist, isoproterenol. Levels of cAMP were detected using the LANCE Ultra cAMP Kit with 30 nM VCP or control siRNA transfected cell or on not transfected cells. Data are representative of 2 independent experiments. B) Western blot analysis of ERK1/2 activation by $\beta_2 AR$ in HEK 293 cells as a response to VCP knockdown. HEK 293 cells were transfected with either 30 nM siRNA VCP or 30 nM siRNA control. 72 hours later, cells were stimulated with 10 μ M isoproterenol or vehicle for 5 minutes. Cells were then lysed and western blot analysis was performed to detect the levels of pERK and total ERK with pERK and ERK-ct antibodies respectively. C) Western blot analysis of ERK1/2 activation by HA-FP in HEK293 cells (stably expressing HA-FP) as a response to VCP knockdown in HEK 293 cells. HA-FP HEK 293 cells transfected with either 30 nM of VCP or control siRNA were stimulated with 1 μ M PGF2 α for 5 minutes. The cells were then lysed and western blot analysis was performed to detect the levels of pERK and total ERK with Anti-ERK/p44/42(T202/Y204) and anti-ERK-ct antibodies respectively. Data are representative of at least three independent experiments.







Figure 22: *Potential Role of ERAD in Regulation of* β_2AR *Signaling Complex Assembly.* **A)** β_2AR association with signalling partners such as G $\beta\gamma$ and adenylyl cyclase (AC) occurs at the level of ER, and this complex traffics to the plasma membrane. Our data suggests that unassembled β_2AR -G $\beta\gamma$ signalling complexes are degraded via ERAD. Mechanisms that recognize and target these receptors for degradation are unknown but we hypothesize that E3 ligases such as gp78 and RNF5 may play a role in ubiquitination (Ub) of the receptor at the level of ER. VCP recognizes ubiquitinated receptors in the ER and retrotranslocates the uncomplexed receptors to the cytosol prior for their degradation via the proteasome. **B)** In a scenario where ERAD is inhibited either by the use of proteasome inhibitors or by knockdown of VCP, the uncomplexed β_2AR targeted for degradation accumulates in the cytosol and/or traffics to the plasma membrane, perhaps in association with other signalling partners. The signalling profile of these receptors is distinct from receptors normally present at the plasma membrane since they activate the pERK pathway as opposed to cAMP/PKA.

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