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Special Section on Phosphoproteomic Analysis of G Protein-Coupled Pathways - Axelrod Symposium—Minireview

Proteomic Approaches to Investigate Regulated Trafficking and Signaling of G Protein–Coupled Receptors

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ABSTRACT

Advances in proteomic methodologies based on quantitative mass spectrometry are now transforming pharmacology and experimental biology more broadly. The present review will discuss several examples based on work in the author's laboratory, which focuses on delineating relationships between G protein–coupled receptor signaling and trafficking in the endocytic network. The examples highlighted correspond to those discussed in a talk presented at the 2019 EB/ASPET meeting, which was organized by Professor Joe Beavo to commemorate his receipt of the Julius Axelrod Award.

SIGNIFICANCE STATEMENT

GPCRs are allosteric machines that signal by interacting with other cellular proteins, and this, in turn, is determined by a complex interplay between the biochemical, subcellular localization, and membrane trafficking properties of receptors relative to transducer and regulatory proteins. The present minireview highlights recent advances and challenges in elucidating this dynamic cell biology and toward delineating the cellular basis of drug action at the level of defined GPCR interaction networks using proteomic approaches enabled by quantitative mass spectrometry.

Introduction

G protein–coupled receptors (GPCRs) make up nature's largest family of signaling receptors and regulate essentially every physiologic process. They also make up a large and important class of therapeutic drug targets, as well as a fundamentally interesting group of integral membrane proteins overall (Hilger et al., 2018). GPCRs were so named for their shared ability to mediate signaling by promoting guanine nucleotide exchange on heterotrimeric G proteins, a family of peripherally associated membrane proteins that operate as intracellular GPCR signal transducers (Sunahara and Insel, 2016). GPCRs also engage a number of other cellular proteins that operate variously as signal transducers, organizers, and regulators. Foremost among these are GPCR kinases (GRKs) and arrestins (Komolov and Benovic, 2018), but there are others, likely including many that remain unknown. Even among known proteins whose interactions with GPCRs are understood in considerable detail (such as G proteins), how these interactions are organized and regulated in intact cells remains incompletely understood.

Proteomic methodologies based on analytical mass spectrometry provide a powerful toolbox for identifying GPCR-interacting proteins and investigating their integrated operation in the complex cellular environment (Lobingier and von Zastrow, 2019). Such approaches are increasingly being applied to the problem of elucidating GPCR signaling and regulation at the level of spatiotemporally organized protein interaction networks in intact cells. The present review will briefly summarize some examples of studies along these lines, focusing on work that was carried out over a period of several years by extraordinarily talented individuals in my laboratory and involving key input from outstanding collaborators. The goal is not to comprehensively elaborate or discuss any particular proteomic methodology in detail, as there are many individuals better qualified to do so and many excellent reviews by such experts [e.g., Domon and Aebersold (2006); Walther and Mann (2010); Willsey et al. (2018)]. Rather, the

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ABBREVIATIONS: APEX, ascorbic acid peroxidase; ASRT, actin and SNX27-associated retromer tubule; B2AR, β2-adrenergic receptor; DOP-R, δ opioid receptor; ESCRT, endosomal sorting complex required for transport; GPCR, G protein–coupled receptor; GRK, GPCR kinase; MOP-R, μ opioid receptor; PDZ, post synaptic density protein, discs large tumor suppressor and zonula occludens-1 protein homology domain; PKA, cAMP-dependent protein kinase; SILAC, stable isotope labeling applied to cell culture; SNX27, sorting nexin 27; TOM1, Target of Myb protein 1; WASH, Wiskott-Aldrich syndrome protein homolog; WWP2, WW domain-containing protein 2.

present review seeks to provide a few specific examples of the application of various methods to address particular questions from the perspective of integrated GPCR cell biology and to discuss current progress in the context of current caveats and future challenges.

GPCR Endocytosis and Signaling Are Intricately Interrelated

A main interest of our laboratory is to understand mechanistic and functional relationships between GPCR signaling and membrane trafficking processes, particularly those involving the endocytic pathway. Accordingly, examples of proteomic approaches highlighted in this review relate primarily to this focus. With this in mind, and to provide some broader perspective on our motivation for applying quantitative proteomic approaches to specific problems in this realm, the following paragraphs briefly summarize a current view of the overall organization of endocytosis and trafficking in the endocytic network after agonist-induced GPCR activation and its relationship to signaling (Fig. 1).

Regulated trafficking starts with agonist-induced endocytosis of GPCRs by clathrin-coated pits. This is controlled primarily at the level of receptor clustering into clathrin-coated pits (von Zastrow and Kobilka, 1992, 1994), with ligand-dependent control conferred through GPCR phosphorylation and binding to β-arrestins (Ferguson et al., 1996; Goodman et al., 1996). Once internalized, individual GPCRs vary considerably in downstream trafficking itinerary, with the itinerary of receptors determined by specific molecular sorting operations (Hanyaloglu and von Zastrow, 2008). A major sorting decision is whether internalized receptors undergo delivery to lysosomes for proteolytic degradation or are nondestructively recycled to the plasma membrane. GPCR sorting between these divergent pathways occurs from a shared endosome intermediate, with the molecular operations determining it being highly specific, because closely

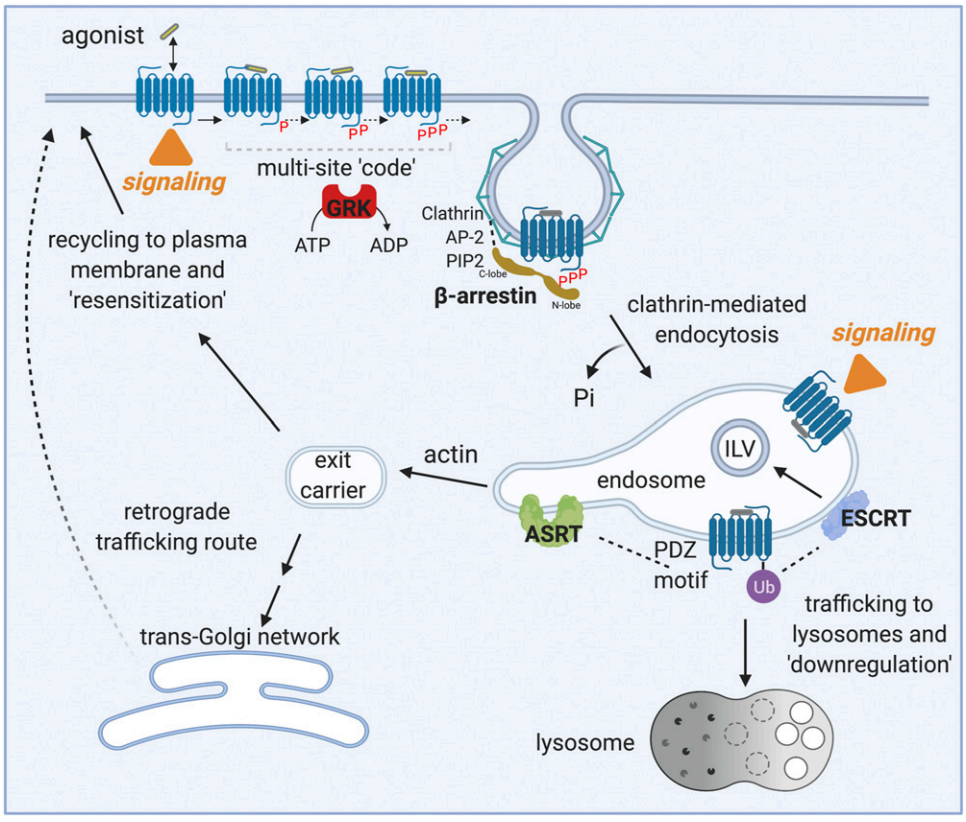

Fig. 1. Summary of major GPCR trafficking-signaling relationships in the endocytic pathway. Agonist-induced activation of the receptor initiates signaling by G protein activation from the plasma membrane. This is followed by agonist-selective phosphorylation by sequential GRK-catalyzed reactions that encode an agonist-selective multiphosphorylation pattern in the receptor tail, which determines whether receptors engage the β-arrestin N-lobe sufficiently strongly to drive receptor clustering in clathrin-coated pits via additional interactions of β-arrestin with clathrin heavy chain, AP-2 (adaptor protein 2), and PIP2 (phosphatidylinositol 4,5-bisphosphate) at the coated pit. This is followed by endocytic scission and packaging of receptors into endocytic vesicles, which then fuse with early endosomes. Receptors are dephosphorylated in endosomes and during endocytic transit, enabling receptors to initiate a second wave of signaling by G protein activation from endosomes. GPCRs are sorted in the limiting membrane by ESCRT, which mediates ubiquitin (Ub)-directed packaging of receptors into vesicles within the endosome lumen; this prevents receptors from recycling and assures their subsequent receptor delivery to lysosomes for proteolytic downregulation. Alternatively, receptors can engage ASRT by a PDZ motif present in the receptor tail, which drives receptor exit from endosomes through actin-dependent formation of endosome-derived carrier vesicles, which can fuse with the plasma membrane or deliver receptors to intermediate recycling endosomes or to Golgi-associated membranes (retrograde route), supporting indirect receptor recycling pathways involving additional sorting. Indicated as orange cones are subcellular locations of known G protein–dependent signal initiation based on present knowledge of the B2AR and opioid receptors. Other GPCRs, such as the thyroid-stimulating hormone receptor and β1-adrenergic receptor, are not presently known to signal from endosomes and, instead, use Golgi-associated membranes as a second site of internal signaling in addition to the plasma membrane (not indicated). All figures included in this review were generated using BioRender (biorender.com). ILV, intralumenal vesicle; Pi, inorganic phosphate.

homologous receptor subtypes, as well as splice variants, can be efficiently sorted separately from one another in the same cells (Tanowitz and von Zastrow, 2003; Tanowitz et al., 2008). Such sorting is also functionally important because it can confer essentially opposite effects on net cellular ligand responsiveness: GPCR trafficking to lysosomes produces a net loss of overall receptor number ("downregulation"), whereas recycling to the plasma membrane promotes a net recovery of functional receptors available at the cell surface ("resensitization") (Law et al., 1984; Pippig et al., 1995).

Our understanding of GPCR signaling-trafficking relationships was limited until recently to such homeostatic control of surface receptor number based on the belief that GPCR signaling is initiated only from the plasma membrane and that internalized receptors are inactive. This view began to change as evidence emerged supporting the hypothesis that β-arrestins act as signal transducers separate from G proteins (Daaka et al., 1998; Luttrell et al., 2018). It has changed even more dramatically over the past several years, with evidence emerging that a number of GPCRs retain, or regain, the ability to activate G proteins after endocytosis (Irannejad et al., 2013) and then use this activity to initiate a discrete "wave" of signal initiation from internal membrane locations after signaling from the plasma membrane (Vilardaga et al., 2014; Irannejad et al., 2015; Calebiro and Koszegi, 2019). Accordingly, the endocytic network is now widely recognized not only to be a site that confers specific homeostatic control over the availability of functional GPCRs at the plasma membrane after prolonged or repeated receptor activation, but also as a discrete and additional location for functional signal initiation by heterotrimeric G proteins and possibly other GPCR-linked intracellular signal transducers.

Use of Quantitative Mass Spectrometry to Elaborate Agonist-Selective Encoding of GPCR Endocytosis by Phosphorylation

GPCR endocytosis by clathrin-coated pits is promoted by the binding of β-arrestin both to activated receptors and to components of the clathrin-associated membrane coat. These interactions, in turn, are typically promoted by activation-dependent phosphorylation of the GPCR cytoplasmic tail mediated by GRKs. This overall mechanism, and the central importance of GPCR phosphorylation in controlling it, were already widely recognized (Kang et al., 2014). However, it was not known whether endocytosis occurs as a consequence of the overall degree of receptor phosphorylation or if there exists a more specific phosphorylation-based "code" in the receptor that determines receptor entry into the endocytic network.

To address this question, we initially focused on the β2-adrenergic receptor (B2AR) because it was well known that ligands differing in relative agonist efficacy at this GPCR produce different amounts of net receptor phosphorylation and internalization. Cells expressing epitope-tagged B2ARs were labeled with "light" (hydrogenated) or "heavy" (deuterated) Leu by the method of stable isotope labeling applied to cell culture (SILAC), incubated in the absence or presence of a selected agonist, and purified from combined cell lysates by epitope tag–assisted affinity isolation. Isolated receptors were then subjected to limited proteolysis followed by enrichment for phosphorylated peptides by metal-affinity purification and then analyzed by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (Trester-Zedlitz et al., 2005). Using this method, several phosphorylations were identified in a region of the B2AR cytoplasmic tail that had been implicated previously by others (by site-directed mutagenesis) in the control of agonist-induced desensitization and internalization of the B2AR (Hausdorff et al., 1991). A particularly interesting observation was that isoproterenol, a catecholamine full agonist that strongly stimulates endocytosis of the receptor, produced phosphorylation at more than one Ser/Thr site in this defined tail sequence. However, dopamine, a weaker agonist that drives overall phosphorylation less strongly, was found to produce phosphorylation at only a single residue in this sequence. These findings provided an initial clue that endocytosis may be determined by a specific phosphorylation code in the cytoplasmic tail of individual GPCRs rather than by the overall amount of phosphate incorporation in the receptor pool. A key feature of this putative code is the simultaneous phosphorylation of more than one Ser/Thr residue in a limited sequence.

We next applied liquid chromatography–mass spectrometry coupled with electrospray ionization. We also expanded our search to the μ opioid receptor (MOP-R) because of the availability of opioid agonists that differ greatly in relative ability to promote receptor endocytosis, but for which differences in the effect on the overall degree of receptor phosphorylation assessed by metabolic labeling and chemical assays was moderate (Keith et al., 1998). Based on this simple observation, we anticipated that a specific phosphorylation code determining regulated endocytosis of receptors—if it existed—might be easier to discern using this GPCR and the ligands available at that time.

Multiple sites of Ser/Thr phosphorylation were identified in the MOP-R tail, and by again using metabolic labeling by the method of SILAC, clear agonist-selective changes in the abundance of resolved phosphorylated tail species were quantified (Lau et al., 2011). Tandem mass spectrometry defined two particularly interesting Ser/Thr-rich "clusters" in the MOP-R tail, each of which fulfilled the key property predicted to be characteristic of an endocytic "code." Specifically, each of the sequences was phosphorylated at more than one Ser/Thr residue in response to a strongly internalizing agonist (a peptide full agonist ligand ligand), but phosphorylation was largely restricted to a single residue in the cluster after receptor activation by a weakly internalizing agonist (the alkaloid partial agonist morphine).

Using site-directed mutagenesis, one of these sequences—the distal Ser/Thr cluster (STANT)—was found to be specifically required for β-arrestin recruitment and endocytosis of MOP-R in response to endocytosis-promoting agonists. The more proximal cluster (TSST in rodent or TSSN in human MOP-R), despite exhibiting a similar degree of agonist selectivity in undergoing multiphosphorylation, did not detectably affect β-arrestin recruitment to receptors or subsequent endocytosis, further supporting specificity of the multisite phosphorylation code. Moreover, single-point mutations in the STANT sequence were found to inhibit DAMGO-induced endocytosis of MOP-R to a similar degree as multisite mutations, supporting the hypothesis that the endocytic code indeed requires multisite phosphorylation within the STANT cluster. Further, in collaborative studies with the group of Stefan Schulz, phosphospecific antibodies were used to verify

and further define the multiphosphorylation code. GRK-mediated phosphorylation of the proximal Ser (S375) in the STANT cluster was found to be stimulated to a similar degree by agonists irrespective of their ability to drive endocytosis of receptors. However, higher-order phosphorylation on the Thr residues, also by GRKs, was found to be specific to strongly internalizing agonists using phosphorylation at S375 as a priming site (Just et al., 2013). Although mutational analysis failed to reveal an effect of phosphorylation in the proximal (TSST/TSSN) cluster on β-arrestin recruitment or MOP-R endocytosis, the group of John Williams subsequently identified a clear effect of this cluster on a different process of longer-term regulation distinct from receptor endocytosis (Arttamangkul et al., 2019). Accordingly, the encoding of agonist-specific effects on GPCRs by multisite phosphorylation may not be restricted to endocytosis, instead revealing a more general principle for selective GPCR control (Fig. 2).

Use of Affinity Purification Mass Spectrometry to Delineate a Mechanism Mediating Sequence-Directed GPCR Sorting

The traditional understanding of how receptors are sorted between lysosomal and recycling pathways requires receptor ubiquitination and ubiquitin-dependent recognition by endosomal sorting complex required for transport (ESCRT), a multiprotein machinery that assembles on the endosome limiting membrane and packages ubiquitinated proteins into vesicles accumulated in the endosome lumen (Henne et al., 2011). An additional mechanism of GPCR sorting was identified through study of the B2AR (Cao et al., 1999). In this mechanism, receptors undergo active sorting into the recycling pathway by extrusion from the endosome limiting membrane rather than by sorting into the degradative route through transfer to the endosome lumen. Further, receptor recognition is mediated by a PDZ domain–interacting motif located in the cytoplasmic tail of the receptor rather than by receptor-attached ubiquitin isopeptides.

The endosome-associated protein that recognizes the B2AR's PDZ motif, and which initiates the alternate sorting mechanism, was identified as sorting nexin 27 (SNX27) in collaborative studies with the laboratory of Tanja Kortemme (Lauffer et al., 2010). It was already evident that this sorting mechanism also somehow requires dynamic actin polymerization (Cao et al., 1999), and also requires the ESCRT-associated endosomal sorting protein HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) (Hanyaloglu et al., 2005), but more detailed and specific insight into how SNX27 works emerged when quantitative mass spectrometry was applied to the problem of identifying additional SNX27-interacting proteins. Collaborating with colleagues in the laboratory of Nevan Krogan, and using a platform developed by this group for unbiased identification of protein interaction

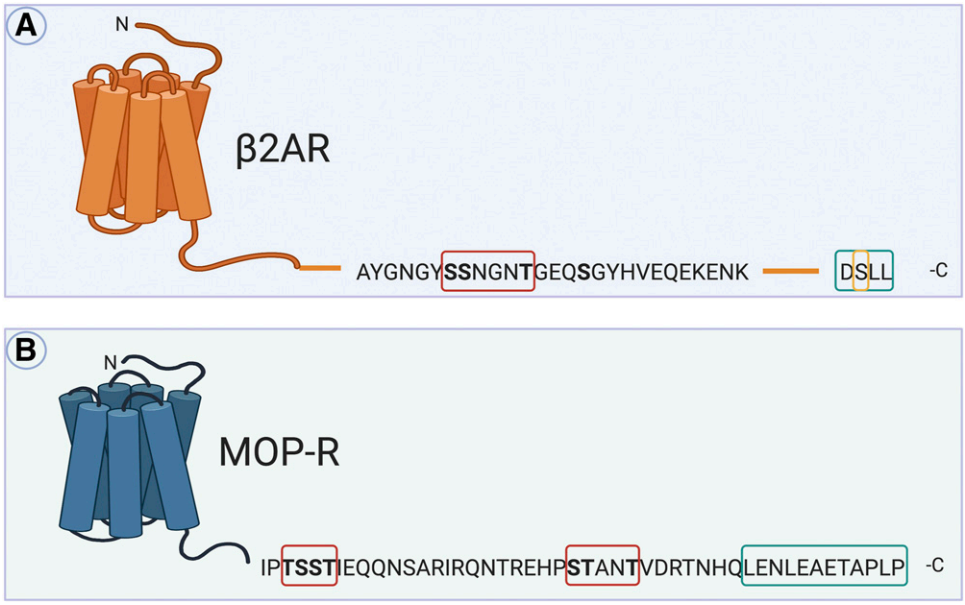

Fig. 2. Overview of key endocytic codes present in the cytoplasmic tails of B2AR and MOP-R. (A) A Ser/Thr-rich sequence in a middle portion of the B2AR cytoplasmic tail is a main site of agonist-induced phosphorylation in intact cells. Agonists that strongly promote endocytosis produce phosphorylation at more than one residue in this sequence, whereas weaker agonists produce phosphorylation on only a single residue. Mutational studies indicate that phosphorylation on more than one of the residues shown in the subregion (red box) is needed for sufficiently strong receptor association with β-arrestin to drive receptor clustering in coated pits for subsequent clathrin-mediated endocytosis of receptors. Also shown is the core PDZ motif present in the distal B2AR tail that drives receptor engagement with ASRT through binding to SNX27. This interaction is required for efficient recycling of receptors back to the plasma membrane. Although not detected so far in the matrix-assisted laser desorption/ionization–time of flight mass spectrometry analysis of receptors isolated from intact cells, the Ser residue in this PDZ motif (boxed in yellow) is a potential site for phosphorylation by GRK5, and phosphorylation of this residue has been observed using tandem liquid chromatography–mass spectrometry. Phosphorylation of this residue effectively disables the recycling sequence by destabilizing its binding to SNX27, resulting in a "switch" of receptor endocytic itinerary from recycling to lysosomal pathways. (B) Two Ser/Thr-rich clusters were identified as main sites of agonist-selective phosphorylation in the MOP-R cytoplasmic tail. Multisite phosphorylation in the STANT cluster (red box) is produced by agonists that strongly promote receptor endocytosis and is required for receptor clustering with β-arrestin in clathrin-coated pits. Agonists that drive endocytosis weakly primarily produce phosphorylation of the STANT cluster on a single residue. A similar agonist-selective trend is observed in phosphorylation of the more proximal TSST cluster (also boxed in red). Phosphorylation of this cluster does not detectably affect endocytosis of MOP-R, however, although it does have other long-term regulatory effects.

partners, multiple components of the WASH complex were identified as interactors with an epitope-tagged version of SNX27 in intact cells (Temkin et al., 2011). WASH is a protein complex associated with the endosome limiting membrane that regulates actin nucleation on endosomes. This was intriguing because, in an earlier live imaging study of PDZ-directed recycling, receptor exit from endosomes was shown to occur from a subset of endosomal tubules associated with dynamic actin polymerization (Puthenveedu et al., 2010). Also emerging from the interaction screen were multiple components of a distinct protein complex called retromer, which associates with the endosome limiting membrane to form tubular membrane evaginations. Through a series of experiments involving coimmunoprecipitation and live cell imaging, WASH and retromer were shown to coassemble on the limiting membrane and form a tubular, SNX27-associated domain on the endosome limiting membrane that we called actin and SNX27-associated retromer tubule (ASRT). Receptors are selectively sorted into ASRT domains by PDZ motif–directed lateral partitioning mediated by SNX27. These tubules appeared to be stabilized by actin assembled around them, but they also formed small transport carriers by membrane scission events that occur at sites flanked by polymerized actin. Retromer was shown previously by others to function in the process of delivering selected membrane proteins from endosomes to Golgi-associated membranes (retrograde pathway) (Bonifacino and Hurley, 2008). Our results established a role of retromer in delivering GPCR cargoes directly back to the plasma membrane. In collaboration with the group of Roger Nicoll, we verified direct endosome-to-plasma membrane by ASRT in neurons and showed that it mediates a local route for rapid delivery of neurotransmitter receptors to the surface of synapses (Choy et al., 2014). Receptor delivery to Golgi-associated membranes, the originally described function of retromer in membrane traffic, was also shown to be possible from ASRT domains (Varandas et al., 2016). However, in contrast to delivery to the plasma membrane that occurs by direct fusion of ASRT-generated carriers, receptor delivery to Golgi membranes requires an additional downstream sorting step (Fig. 1). Subsequent work by the laboratory of Peter Cullen, carried out independently of our group but also using quantitative proteomics, provided additional insight to the operation of this PDZ motif–directed recycling mechanism and expanded its range of cargoes beyond receptors (Steinberg et al., 2013).

Use of APEX-Mediated Proximity Labeling and Quantitative Mass Spectrometry to Spatiotemporally "Map" Cytoplasmic Protein Interactions with GPCRs

A limitation of protein interaction analysis using affinity purification mass spectrometry is that many important interacting proteins are likely missed because the interactions are transient in the cell or are not sufficiently stable to be retained after cell lysis. Various methods have been developed to address this problem, and among them, proximity labeling offers considerable promise. A particular challenge in studying GPCR trafficking and signaling with such methods is the speed with which receptors move within cells, causing the overall protein environment around receptors to change. To address this challenge, our laboratory initiated a collaborative

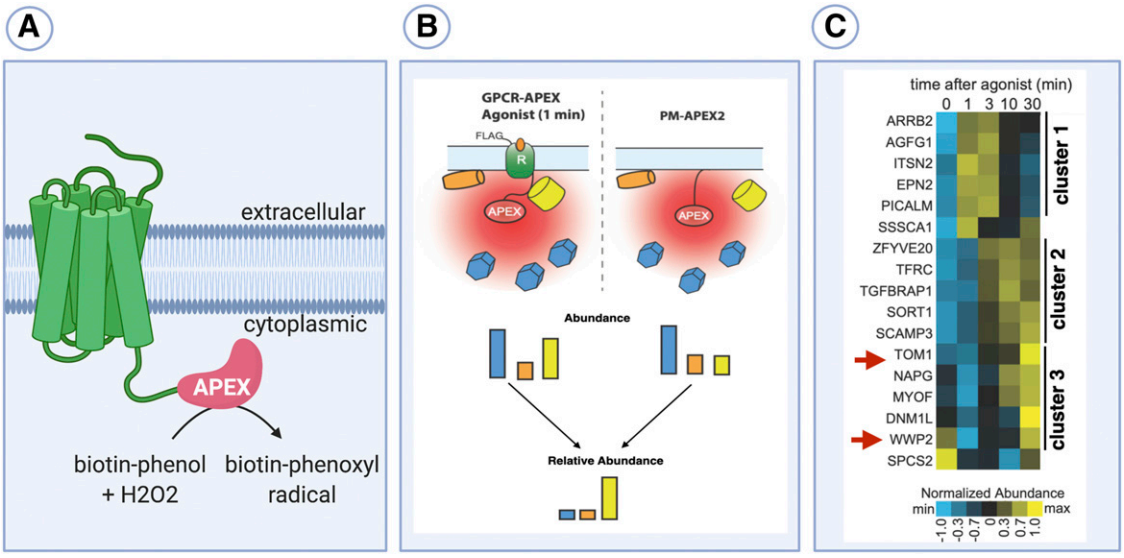

Fig. 3. Outline of APEX-based proteomic strategy applied to GPCRs. (A) General strategy for proximity labeling by GPCRs based on fusion of APEX2 to the cytoplasmic receptor tail and initiating labeling by preincubation of cells with biotin phenol followed by acute application of hydrogen peroxide. APEX2 catalyzes the production of biotin-phenoxyl radicals that dissipate by diffusion. (B) Overview of strategy for deconvolving bystander and direct interactions using location references. Proximity labeling mediated by the local "cloud" of biotin-phenoxyl radicals produces labeling of proteins preferentially within a radius of ~20 nm. This region includes specific interactors but also "bystander" proteins that are located in sufficiently close proximity or diffuse through the biotin-phenoxyl cloud during the labeling reaction. The proteomic data resulting from such labeling represents the product of the consequences of direct interaction with receptors convolved with such bystander effects. The proteomic signal can be deconvolved mathematically using spatial references, provided that the quantitative proteomic data are of sufficiently high quality. (C) The result of proteomic analysis of APEX proximity labeling fused to MOP-R, clustered as a function of time after application of the MOP-R agonist DAMGO. Two proteins that are enriched after 30 minutes of agonist exposure are WWP2 and TOM1. As discussed in the text, both of these proteins were functionally validated based on knockdown studies demonstrating that both WWP2 and TOM1 are required for sorting of internalized MOP-R to lysosomes.

effort with the laboratories of Alice Ting and Nevan Krogan to capture "snapshots" of protein interaction networks engaged by GPCRs based on rapid proximity labeling using an engineered ascorbic acid peroxidase enzyme developed by the Ting group fused to the receptor (Fig. 3A) and applying quantitative proteomics combined with a system of spatial references to mathematically "deconvolve" the complex protein labeling data to distinguish "bystander" and "specific" interactors (Fig. 3B).

Using this approach, a number of previously known protein interactions with the B2AR were verified and mapped to their known subcellular locations (Lobingier et al., 2017). Then, the approach was applied to unbiased analysis of protein interactions engaged by the δ opioid receptor (DOP-R). This is an enigmatic GPCR because it is sorted to lysosomes by a mechanism that requires ESCRT but does not require direct ubiquitination of receptors. The deconvolved APEX data stream identified a number of novel candidate interactors with DOP-R in endosomes, including the ubiquitin ligase WWP2 and ubiquitin-binding protein TOM1 (Fig. 3C). Functional analysis indicated that both WWP2 and TOM1 are essential to enable internalized DOP-Rs to undergo ESCRT-dependent delivery to lysosomes. Previous work suggested the existence of alternate, ubiquitination-independent protein connectivity to ESCRT mediating DOP-R delivery to lysosomes. The APEX data suggest that, in addition, the sorting of wild-type DOP-R (which normally does undergo ubiquitination) involves receptor interaction with a specific ubiquitin ligase (WWP2) and a distinct ubiquitin-binding protein (TOM1) that is closely related to the ESCRT-associated sorting protein HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) . Together, these results provide additional insight to the complexity of GPCR sorting in the endocytic network and support the validity of the APEX method as a means to achieve unbiased identification of new GPCR interaction partners.

Use of Phosphoproteomic Profiling to Investigate Endosomal Signaling by GPCRs

Another application of mass spectrometry–based proteomics in GPCR cell biology is unbiased profiling of signaling effects downstream of the receptor. Signaling by the B2AR provides an example because this GPCR activates cAMP production by coupling to Gs-family heterotrimeric G proteins, a major downstream effector of which is the cAMP-dependent protein kinase (PKA). Previous work had shown that activation of cAMP production from endosomes is necessary for efficient induction of cAMP-dependent gene expression by endogenous B2AR activation, relayed by phosphorylation of the cAMP-dependent response element binding protein (Tsvetanova and von Zastrow, 2014). However, it was not known whether the effect of endocytosis on phosphorylation by PKA is specific to cAMP-dependent response element binding protein, restricted to nuclear PKA targets, or represents a more general selective signaling phenomenon involving cytoplasmic targets. To address this, cells were metabolically labeled for quantification by SILAC, and liquid chromatography–mass spectrometry was performed after phosphopeptide enrichment to assess what effect endocytic blockade has on the overall phosphoproteomic signature of endogenous B2AR activation in intact cells. The results revealed a mixed picture, with some cytoplasmic targets highly sensitive to endocytic blockade and others less so, but most targets exhibited at least some preference for phosphorylation by cAMP produced from endosomes relative to the plasma membrane. An interesting additional observation was that even agonists that drive endocytosis of the B2AR relatively weakly can produce endocytosis-dependent phosphorylation of a number of PKA targets. This suggests that endosomes represent favored sites for cAMP signaling by PKA in general, with cAMP production from endosomes producing a highly sensitive response (Tsvetanova et al., 2017).

Conclusion and Future Directions

The studies summarized above provide several examples of the application of both established and emerging proteomic methodologies to the study of GPCR cell biology, with a particular focus on investigating relationships between receptor membrane trafficking in the endocytic network and cellular signaling mediated by selected GPCRs. These are certainly not isolated examples, as quantitative proteomic methods involving protein mass spectrometry have been successfully applied to the study of many other biologic processes that are beyond the scope of the present review to discuss. Accordingly, the present examples provide only a limited sampling of experimental possibilities and are focused on specific cell biologic questions. Despite this admittedly narrow view, it is already clear that proteomic methods have the potential to transform cell biology and enable incisive elucidation of complex regulatory mechanisms in the intact cellular environment, and they are able to do so with increasingly refined spatiotemporal resolution.

Many challenges remain, however. In particular, proteomic approaches remain limited both in detection sensitivity and specificity, with the problem of nonspecific detection particularly acute using rapid proximity labeling. Accordingly, care is required in designing experimental strategies with sufficient internal controls to reduce "noise" due to nonspecific interactions or labeling and to externally validate candidate interactions using additional methods. Accordingly, we view modern advances in quantitative proteomics as a box of powerful tools that must be used with care and, typically, in combination with other methods. When used appropriately, however, the proteomic toolbox enables inroads to be made into areas of mechanistic cell biology that had previously been resistant to incisive experimental interrogation or were not even known to exist.

We think that a particularly exciting direction of future investigation is toward improved methods for spatiotemporal resolution of protein interaction networks with GPCR in living cells and in real time. By making continued progress in this area, we anticipate that it will become possible to approach a biochemical elucidation of complex biologic processes more closely as they occur in vivo—arguably the ultimate goal of mechanistic cell biology. We also believe that further application of proteomic methods to delineate ligand-selective regulation of GPCRs is a most interesting future direction that may have particularly important therapeutic implications. For example, it is now clear that chemically distinct agonists indeed have the potential to selectively "encode" distinct regulatory effects by producing different patterns of multisite

phosphorylation in the receptor. We anticipate that this principle will open a new path toward improving the specificity and efficacy of GPCR-directed therapeutics based on manipulating the encoding scheme with drugs. In addition, with continued advances in understanding mechanisms and consequences of initiating GPCR signaling reactions from internal membrane locations relative to plasma membrane, it may be possible to further improve the precision of GPCR therapeutics.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: von Zastrow.

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