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MEMBRANE TRAFFICKING OF G PROTEIN–COUPLED RECEPTORS

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■ **Abstract** G protein-coupled receptors (GPCRs) modulate diverse physiological and behavioral signaling pathways by virtue of changes in receptor activation and inactivation states. Functional changes in receptor properties include dynamic interactions with regulatory molecules and trafficking to various cellular compartments at various stages of the life cycle of a GPCR. This review focuses on trafficking of GPCRs to the cell surface, stabilization there, and agonist-regulated turnover. GPCR interactions with a variety of newly revealed partners also are reviewed with the intention of provoking further analysis of the relevance of these interactions in GPCR trafficking, signaling, or both. The disease consequences of mislocalization of GPCRs also are described.

INTRODUCTION

Extensive investigation has revealed several mechanisms that modulate G protein-coupled receptor (GPCR) responsiveness following agonist occupancy, including agonist-elicited receptor desensitization, endocytosis, and resensitization (or degradation, depending upon the individual GPCR). In fact, a particularly active area of research at present is trying to understand the linkage between receptor endocytosis and receptor-evoked signaling (1). However, less attention has focused on the molecular determinants accounting for how receptors achieve specific localization in the agonist-naïve state. Appropriate receptor localization on the cell surface is required to permit access to requisite ligands and signal transduction machinery. Cell surface localization is governed by two predominant mechanisms: receptor delivery to a particular site and retention at that site. The functional importance of GPCR localization is emphasized by diseases that ensue from receptor mislocalization, such as retinitis pigmentosa (2); X-linked nephrogenic diabetes insipidus (NDI) (3); and hypogonadotropic hypogonadism (4), which result from intracellular accumulation of mutant rhodopsin, the V2 vasopressin receptor (V2R), or the gonadotropin releasing hormone (GnRH) receptor, respectively (see below).

This chapter addresses four fundamental properties of GPCR trafficking: (*a*) appropriate delivery of nascent GPCRs to the cell surface; (*b*) mechanisms for retention of GPCRs at the cell surface; (*c*) turnover of GPCRs at the cell surface, including agonist-regulated GPCR turnover, and (*d*) diseases that ensue from disruption of cell surface localization.

DELIVERY OF NASCENT GPCR TO THE CELL SURFACE

Alpha$_2$-Adrenergic Receptor Subtypes as a Model System

Our studies have exploited the individual α_2-adrenergic receptor (AR) subtypes as models for characterization of GPCR targeting and localization owing to their unique trafficking itineraries in both polarized and nonpolarized cells. In vivo, α_2-ARs are found in epithelial cells of the renal proximal tubule and proximal colon (5–7). Functional and immunocytochemistry studies show that the α_2-ARs are concentrated on the basolateral surface of these cells. Polarized Madin Darby canine kidney (MDCK) II cells have been employed as a cellular system to examine the mechanisms of polarized protein targeting in general (8–11); we have exploited MDCK II cells to evaluate the trafficking properties of α_2-AR subtypes, in particular, as a model system relevant to all GPCRs. MDCK II cells, although not derived from the proximal tubule, polarize functionally and morphologically in culture. Because a great deal has been learned about trafficking of non-GPCR signaling molecules, relying on these cells as a model system allows investigators to compare and contrast findings. For studies of the α_2-AR subtypes, epitope-tagged receptors were heterologously expressed in MDCK II cells. At steady state, all three α_2-AR subtypes reside on the basolateral surface in MDCK II cells, analogous to their localization in epithelial cells in vivo. However, the α_2-AR subtypes achieve this basolateral localization via different trafficking itineraries. Whereas the α_{2A}-AR and α_{2C}-AR are targeted directly to the basolateral surface, the α_{2B}-AR is randomly distributed to both the apical and basolateral surfaces and then selectively retained at the lateral subdomain (12, 13).

Intentional mutagenesis and truncation studies have shown that the signals required for appropriate α_{2A}-AR targeting to the basolateral surface of MDCK II cells reside within transmembrane or membrane-adjacent regions of the receptor. Mutations that result in the elimination of the third intracellular loop, truncation of the carboxyl terminal, or modification of the tyrosine or phenylalanine endocytosis motifs do not alter direct basolateral surface delivery (12–14). Moreover, α_{2A}-AR basolateral trafficking occurs independently of posttranslational receptor modifications; α_2-ARs mutated to eliminate acylation and glycosylation nonetheless achieve proper basolateral localization. Consistent with the interpretation that targeting information resides within membrane-embedded sequences of the α_{2A}-AR is that an α_{2A}-AR mutant structure that represents the hydrophobic core of the receptor retains proper trafficking to the basolateral surface (12).

To elucidate the regions within the transmembrane domains encoding basolateral surface targeting information, truncations of the α_{2A}-AR and chimeras with the apically targeted A1 adenosine receptor were evaluated. These studies revealed that α_{2A}-AR targeting to the basolateral surface relies upon multiple, noncontiguous, membrane-embedded sequences within or near the lipid bilayer, and suggested that a three-dimensional surface, as opposed to a single linear sequence, provides the basis for interaction with trafficking molecules (15).

Similarly, the α_{2C}-AR is directly targeted to the basolateral surface and exhibits a similar residency time on this membrane comparable to the α_{2A}-AR and α_{2B}-AR subtypes [$t_{1/2} = 10$–12 h (13)]. However a substantial fraction of the α_{2C}-AR remains intracellularly in a large, nonrecycling pool when studied in polarized (13) and nonpolarized cells (16–22). Interestingly, this intracellular pool is not observed when murine α_{2C}-ARs are expressed in rat PC12 cells. These findings suggest that rodent α_{2C}-AR in cells of neural crest origin may interact with proteins uniquely expressed in these cells that are responsible for tethering the receptor to the cell surface (17, 18, 22). Cell growth at lower temperatures (28°C) also results in α_{2C}-AR redistribution to the cell surface. These findings suggest that the intracellular pool of α_{2C}-AR may accumulate for thermodynamic reasons, such that the α_{2C}-AR may assume multiple conformations during processing, only some of which are productive for cell surface delivery. Reduced temperature may allow the α_{2C}-AR to assume conformations that more facilely traffic to the surface (23, 24).

In contrast to the direct delivery of the α_{2A}-AR and α_{2C}-AR subtypes to the basolateral surface in polarized MDCK II cells, the α_{2B}-AR subtype is delivered randomly to both the apical and basolateral surfaces but is selectively retained on the basolateral membrane, based on studies utilizing metabolic [^{35}S]Cys-Met labeling of polarized MDCK II cells followed by selective surface membrane biotinylation (13). The apically targeted α_{2B}-AR displays a rapid turnover on this surface ($t_{1/2} = \sim$minutes), whereas the basolateral α_{2B}-AR demonstrates a prolonged surface residence [$t_{1/2} = 8$–10 h (13)], which accounts for the observation that at steady state the α_{2B}-AR is detected only on the basolateral membrane using immunocytochemical strategies. The random delivery of the α_{2B}-AR to both surfaces has been exploited to reveal the role of spinophilin in basolateral retention of the α_2-AR subtypes. Thus, redirection of a domain of spinophilin that interacts with α_2-ARs to the apical surface extends the half-life of the randomly delivered α_{2B}-AR on that surface and provides one line of evidence that the basolateral retention of α_2-AR subtypes involves its interaction with spinophilin. Spinophilin, a multidomain protein, normally underlies the basolateral surface as part of the E-cadherin-induced cytoskeleton (25) and has been shown to interact with G_i/G_o GPCRs, including the α_2-AR subtypes.

Subtype-specific differences in α_2-AR subtype trafficking in the same cellular background, e.g., MDCK II cells, support the notion that information within the receptor protein, rather than exclusively in the cellular background, provides the information for appropriate delivery and retention. Moreover, these models provide an opportunity to explore the role of the various receptor domains, as well as

various receptor-protein interactions, potentially involved in GPCR targeting and retention.

Motifs Implicated in GPCR Trafficking to the Cell Surface

A variety of amino acid motifs, particularly in predicted endofacial domains of GPCRs exposed to the cell interior, have been implicated in constitutive and agonsit-evoked endocytosis of GPCRs. These are discussed below.

TYROSINE-BASED MOTIFS These motifs [YXXϕ or NPX(n)Y; where Y = Tyr, N = Asn, P = Pro, X = any amino acid, ϕ = any bulky hydrophobic amino acid] have been implicated in the protein trafficking of numerous single- or multi-transmembrane-spanning proteins (26). The involvement of the YXXϕ motif in protein endocytosis, internalization, lysosomal targeting, and Golgi network trafficking has been demonstrated (27). For example, proper basolateral targeting of the aquaporin4 water channel is dependent upon the YXXϕ motif, as disruption of this motif results in defective channel localization (28). Similarly, the importance of this motif is observed for some single-transmembrane-spanning receptors, such as the low density lipoprotein (LDL) and the transferrin receptors, which interact via their YXXϕ motifs with specific adaptor proteins, such as the μ1B subunit of the AP1 adaptor complex, to achieve basolateral membrane localization in polarized epithelial cells (29, 30).

Characterization of the NPX(n)Y motif in various single- or multitransmembrane-spanning proteins also reveals its diverse role in mediating internalization and targeting (31–34). A role for the NPX(n)Y motif in trafficking of membrane proteins (particularly in surface targeting as well as endocytosis from the surface) prompted our evaluation of the impact of this sequence on the delivery of the α_{2A}-AR to the basolateral surface of polarized MDCK II cells. Mutation of Tyr 426 (Y426) to Ala (A426) did not perturb direct basolateral surface delivery (12). In many GPCRs, the NPX(n)Y motif is followed by a YTRF (where T = Thr, R = Arg, F = Phe) motif, which is implicated in transferrin receptor endocytosis (35). Similarly, mutation of Phe 429 (F429) to Ala (A429) in the α_{2A}-AR did not perturb basolateral targeting. However, simultaneous mutations of the Y426 and F429 residues to create NPX(n)ATRA resulted in a receptor that accumulated intracellularly, perhaps owing to receptor misfolding. Nonetheless, the lack of impact by individual mutations suggests that neither the NPX(n)Y nor YTRF sequences are critical for direct basolateral delivery of the α_{2A}-AR subtype to the surface of polarized MDCK II cells. Like the α_{2A}-AR, mutation of Y324 in the NPX(n)Y motif of the gastrin-releasing peptide receptor does not alter cell surface delivery, binding, or turnover (35a). In some cases, normal surface expression, but perturbed ligand binding, occurs following mutation of the tyrosine residue within the NPX(n)Y motif, as seen for the somatostatin receptor type 5 [hSSTR5-Y304A (36)] or the CB2 cannabanoid receptor [CB2-Y229A (37)]. Alternatively, there are GPCRs for which mutation of the tyrosine residue in the NPX(n)Y motif manifests altered

effector coupling characteristics despite normal surface expression and retention of ligand binding, e.g., platelet-activating factor [PAF-Y293A (38)], α_{1B}-AR [α_{1B}-AR-Y348A (39)], and angiotensin II type 1 [AT_{1A}R-Y302A or Y032F (40)].

A role for the NPX(n)Y motif in agonist-evoked endocytosis has been documented for the β_2-AR under some experimental circumstances (41). Disruption of the NPX(n)Y motif of the β_2-AR (β_2-AR-Y326A) does not appear to perturb delivery to the cell surface, coupling to adenylyl cyclase, or rapid agonist-induced desensitization when receptors were overexpressed at [$\sim$1 pmol/mg (41)]. However, when receptor density is reduced ($\sim$200–300 fmol/mg), high-affinity β_2-AR-Y326A binding and adenylyl cyclase coupling is lost (42), probably because the majority of these mutant receptors are retained intracellularly; these investigators interpret their findings as a demonstration that the β_2-AR Y326A achieves steady state intracellular accumulation owing to constitutive sequestration of endocytosed receptors (41, 42). Similar observations have been documented for the Neurokinin 1 receptor (43).

The different consequences of mutating the NPX(n)Y motif in a variety of GPCRs may be reflective of the highly conserved nature of this motif among different GPCRs and suggest an important structural role for this sequence. It is possible that structural perturbations create a less stable receptor structure, which leads to differing functional outcomes (i.e., alterations in trafficking, ligand binding, effector coupling, or receptor internalization), depending on the particular receptor structure.

DILEUCINE MOTIFS Dileucine (L-L or LXL, where L = Leu) motifs also have been found to mediate diverse functions, including internalization and targeting (28, 44–48), suggesting that these motifs may mediate multiple important spatial and temporal spatial tasks. The involvement of a dileucine motif (or a dihyrophobic motif in general) in GPCR trafficking has been studied most extensively with the V2R (49). The V2R contains a dileucine motif (L339, L340) immediately preceding dicysteine palmitoylation sites. The naturally occurring mutant R337X V2R is truncated two residues upstream of this dileucine cluster and, when expressed heterologously, is retained intracellularly (49–51). Radioligand binding assays demonstrate, at least for the V2R, that Leu 339 and Glu 335 play dominant roles (and Leu 340 to a lesser extent) in cell surface expression (52). Cells expressing V2Rs mutated at V2R-E335G-GFP, V2R-L339T-GFP fused to green fluorescent protein, or a double-leucine mutant (V2R-L339/340T-GFP), demonstrate fluorescence accumulation within the endoplasmic reticulum (ER) at steady state. These data support a role for the V2R carboxyl-terminal dileucine motif in facilitating proper receptor maturation through the ER/Golgi complex, an observation also reported for the luteinizing hormone/chorionic gonadotropin receptor (53). For the CXCR4 receptor, in contrast, the dileucine motif plays an important role in both phorbol myristyl-acetate- and agonist-promoted internalization (53a).

In contrast to findings for the V2R, mutation of the β_2-AR and V1a vasopressin receptor dileucine motif does not alter receptor delivery to the cell surface;

receptors with mutant dileucine motifs display wild-type-like radioligand binding and normal receptor-elicited effector coupling (54, 55). However, the singly mutated β_2-AR-L339A and β_2-AR-L340A, or the double mutants β_2-AR-L339/340A and V1aR-L361/362A, fail to undergo agonist-stimulated endocytosis, suggesting a role for the dileucine motif in receptor internalization (54, 55). Interestingly, this is somewhat similar to the diversity of phenotypes observed for palmitoylation-less GPCR mutants. The effect of the dileucine motif on GPCR trafficking may be rate limiting at different partial reactions composing the receptor's itinerary over its lifetime in the cell, depending on the GPCR. A role for dileucine motifs in receptor endocytosis and receptor recycling has been demonstrated for a variety of structural families (56).

FxxxFxxxF MOTIFS Studies in nonpolarized cells have shown that the FxxxFxxxF motif found in the carboxyl tails of the D1R (D1R), the M2 muscarinic receptor, and the angiotensin II AT_{1A} receptor appears necessary for cell surface expression (57, 58). Yeast two-hybrid analysis and GST-fusion pull down assays demonstrated that the FxxxFxxxF motif found within the carboxyl region of the D1R interacts with a novel protein of molecular mass 78 KDa named DRiP78, an interaction that is critical for surface localization. Mutation of F333, F337, and F341 of the D1R results in endoplasmic reticulum retention, loss of cell surface expression, radioligand binding, and effector coupling. Consistent with a role of DRiP78 in trafficking D1R to the cell surface, overexpression of a carboxyl-terminal DRiP78 peptide that competes with full-length DRiP78 for binding to the D1R reduces cell surface receptor localization (57). Similar observations were observed with the M2 muscarinic receptor and the angiotensin II AT_{1A} receptor (57, 58). Taken together, these data suggest that DRiP78 may function as an ER export protein that facilitates receptor maturation by recognizing the FxxxFxxxF motif found within the carboxyl region of GPCRs.

BASIC DOMAINS Basic domains have been implicated in the cell surface expression of the chemokine receptor (59) as well as in targeting mGluR1a and mGluR1b splice variants of the mGluR1 receptor to distinct domains in polarized epithelial cells and neurons (60, 61). Whereas the mGluR1a variant targets to dendrites in neurons and to the basolateral surface in polarized renal epithelial cells, the mGluR1b variant is found axonally in neurons and apically in polarized cells. Alternative splicing of the mGluR1 results in a long ($>$300 amino acid) carboxyl tail for the mGluR1a and a short ($\sim$20 amino acid) carboxyl tail for the mGluR1b receptor (62). Although both receptor carboxyl termini contain the apical targeting RRK motif (amino acids 887–879), this sequence appears to dictate receptor localization only for the MGluR1b splice variant. Receptor chimera studies reveal that the mGluR1a contains a dominant basolateral targeting motif (amino acids 1012–1071) that overrides the RRK apical signal, accounting for mGluR1a receptor variant redirection to the dendritic/basolateral domains (60, 61).

Masking of apical targeting signals by basolateral determinants occurs for other GPCRs when expressed in polarized cells, including the V2R (63), the follicle-stimulating hormone receptor (64), and the M3 muscarinic receptor (65). Interestingly, despite deletion of the carboxyl-tail basolateral signaling sequence, the M3AchR still targets basolaterally. This finding suggests that despite the strong influence of this motif (as assessed by redirecting apically targeted proteins to the basolateral membrane), other sequences likely also contribute to the basolateral targeting, reminiscent of observations with the α_{2A}-AR (12, 14, 65).

As described above, some GPCRs reside on the apical surface of polarized cells. A1 adenosine receptors (66) and rhodopsin are targeted directly to the apical surface. In the case of rhodopsin, fusion of its carboxyl terminus to CD7, a cell-surface epitope defined in immune-responsive cells that normally trafficks to the basolateral surface of polarized cells, redirects the basolateral targeting of CD7 to the apical domain in polarized epithelial MDCK II cells (67). Similarly, the carboxyl termini of the apically targeted serotonin 5-HT_{1B} (68) and metabotropic mGluR7 (69) also contain apical targeting determinants that permit polarized expression of GPCRs.

POLYPROLINE MOTIFS Polyproline motifs are known to mediate protein-protein interactions with Src homology SH3 domains (70). This motif has been found in a variety of GPCRs, including the β_1-AR, β_3-AR, α_{2A}-AR, and D4 dopamine receptor. For the D4 dopamine receptor and β_1-AR, proline-rich regions of sequence have been demonstrated to interact with multiple SH3 domain-containing proteins, including Nck and Grb2 for the D4 dopamine receptor (71) and endophilins (SH3p4, p8, and p13, also known as endophilin 1, 2, and 3, respectively). Studies on the β_1-AR indicate that the endophilins play a role in defining the interplay between β_1-AR-G_s coupling versus agonist-induced internalization, the latter of which is enhanced by overexpression of endophilin 1 (SH3p4) (72).

As can be seen from the numerous examples discussed above, the presence of a particular motif (or multiple motifs) in a GPCR sequence does not necessarily dictate a predicted targeting regimen. Clearly, demonstrating the causal role of a particular sequence in polarized trafficking of GPCRs requires direct testing for each GPCR. The ability of one targeting sequence to override another in determining the localization of GPCRs also may result from differential interaction of GPCRs with various accessory or regulatory proteins, as discussed below.

Interactions of GPCRs with Accessory Molecules Can Influence Cell Surface Delivery

GPCRs have been shown to interact with numerous proteins, some of which play already demonstrated roles in cellular trafficking, such as serving as molecular chaperones to assist in the folding, processing, or cellular transport of GPCRs through the secretory pathway. Additionally, oligomerization of GPCRs with

cognate family members has emerged as an important contributing factor in receptor localization and function, with $GABA_B$ receptors serving as a key example.

GPCR OLIGOMERS IN RECEPTOR TRAFFICKING The demonstration that rhodopsin, in its native state, is found in ordered, dimeric arrays substantiates the proposed dimeric form of rhodopsin and other GPCRs suggested by extensive biochemical and pharmacological analyses. The reader is referred to several comprehensive reviews examining the discovery and impact of GPCR oligomerization (73–75). Discussion about GPCR oligomerization in this review will focus on its role in GPCR processing to the cell surface.

The role of receptor oligomerization in receptor trafficking initially was revealed for the metabotropic receptor for γ–aminobutyric acid (GABA). GABA, the predominant inhibitory central nervous system neurotransmitter in mammalian systems, mediates its effects via binding to ionotropic ($GABA_{A/C}$) receptors to elicit fast synaptic inhibition or via metabotropic ($GABA_B$) GPCRs to elicit slow, sustained inhibition. $GABA_B$ receptors are in the class III or Family C GPCR family and contain an extensive extracellular ligand binding domain and a large intracellular carboxyl terminus. The $GABA_B$ receptor family is subdivided into $GABA_BR1$ (consisting of two receptor splice variants, $GABA_BR1a$ and $GABA_BR1b$, differing in their extreme amino terminus), and $GABA_BR2$. $GABA_BR1a/b$ and $GABA_BR2$ mRNA distribution overlaps in different neuronal cells and tissues (76, 77).

Several independent investigations have determined that heterodimerization of $GABA_BR1$ and $GABA_BR2$ is necessary for cell surface expression of a functional $GABA_B$ receptor. When the cDNA encoding $GABA_BR1a$ (or $GABA_BR1b$) were individually expressed in ganglion neurons, the proteins expressed were immaturely processed glycoproteins that were retained in the ER and failed to reach the cell surface and mediate effector activation when expressed individually in heterologous systems (78, 79). In contrast, individual expression of the $GABA_BR2$ subtype results in a protein that resides on the cell surface. Although individually expressed $GABA_BR2$ can couple to inhibition of adenylyl cyclase (80), this molecule does not fully recapitulate $GABA_B$ receptor pharmacology in terms of ligand binding affinity and specificity (77, 80, 81). Biochemical and yeast two-hybrid cloning strategies revealed that the $GABA_BR1a/b$ and $GABA_BR2$ proteins interact, which is consistent with the overlapping mRNA distribution of R1 and R2 subunits (79, 81).

Coexpression of both R1 and R2 subunits of $GABA_B$ receptors reconstitutes the specificity and effector activation profile characteristic of the pharmacologically defined $GABA_B$ receptor, i.e., high-affinity radioligand binding, inhibition of forskolin-stimulated adenylyl cyclase, agonist-dependent GIRK channel activation, and agonist-dependent stimulation of $[^{35}S]GTP\gamma S$ binding (76, 77, 79–82). Heterodimerization involves the carboxyl terminus of the individual $GABA_BR1a/b$ and $GABA_BR2$ receptors (83). Further dissection of the carboxyl terminus has revealed an ER retention signal in the $GABA_BR1$ coiled-coil domain (84, 85), which is masked upon heterodimerization of the $GABA_BR1a/b$ and $GABA_BR2$, thus

permitting targeting the heterodimers to exit the ER and traffic to the cell surface (86).

A homology-based bioinformatics approach was employed to identify $GABA_{BL}$, a novel $GABA_B$-related GPCR (85). Expression of the $GABA_{BL}$ alone, or in combination with the R1 or R2 subunits, does not result in expression of a functional GABA receptor; however, these data suggest that another related subunit or chaperone may be required for the surface expression of a functional GABA receptor involving the $GABA_{BL}$ protein (85).

The gustatory system provides yet another example of a role for oligomerization in receptor trafficking and signaling. The mammalian taste system detects five flavor qualities in taste buds: bitter, sweet, salty, sour, and umami (the taste of glutamate). Different flavors stimulate G protein-coupled taste receptors (TRs) expressed on cells of the tongue. The taste receptor GPCR family is defined by the sweet/umami T1Rs (consisting of the T1R1, T1R2, T1R3 variants), and the bitter T2Rs [corresponding to > 30 members (87)]. Interestingly, TRs are distantly related to $GABA_B$ receptors (88). Functional complementation data are consistent with the interpretation that T1Rs heterodimerize among their subtypes to support the various taste qualities. For example, the T1R1/T1R3 heterodimer detects umami tastes, whereas the T1R2/T1R3 heterodimer responds to sweet tastes (89). Mice harboring the T1R3 polymorphism (T1R3 I60T) are refractory to sweet tastes (the nontaster allele). Structural modeling predicts that T1R3 I60T creates a site for O-linked glycosylation that could perturb dimerization (90), although it has been observed that the T1R3 I60T allele does coimmunoprecipitate with the T1R2 (and T1R1) allele when overexpressed in heterologous cell systems (88). What remains to be established rigorously is whether taste insensitivity concomitant with expression of the T1R3 I60T allele is due to perturbed trafficking of functional taste receptors (monomers or oligomers) to the cell surface, loss of binding and/or signaling capability, or a combination of molecular dysfunctions.

Bioluminescence resonance energy transfer (BRET) and coimmunoisolation studies have revealed that oligomerization of the V1a, V2 vasopressin, and oxytocin receptors occurs early in ER processing even before the receptor acquires an Endo H–resistant glycosylation state (91). Numerous mutants identified for the V2 vasopressin (92), opsin (93), gonadotropin (94), calcium sensing (95), $GABA_B$ (96), CCR5 chemokine (97), and D3 dopamine (98) receptors have been documented to negatively regulate wild-type receptor expression and/or function. Cumulatively, these data support the concept that dimerization is constitutive (i.e., independent ligand binding at the cell surface), or at least a general property of a majority of GPCRs, which may occur early in receptor synthesis and may be an important determinant in wild-type receptor maturation and ultimately in ligand specificity and cellular signaling.

For some GPCRs, heterodimerization appears to be important for pharmacological specificity once at the cell surface but not for cell surface expression. For example, κ- and δ-opioid receptors (KOR and DOR, respectively) individually are capable of properly targeting to the plasma membrane, binding ligand,

and coupling to predicted effectors (99), although it is likely that the homodimers of these receptors are reaching the cell surface. Coimmunoprecipitation from cells expressing the two subtypes confirms KOR and DOR heterodimerization; however, pharmacological assessment of the heterodimer reveals properties distinct from those seen when assessed for the individual receptors. This phenomenon also has been observed for other GPCRs, including the μ-(MOR) and δ-opioid receptors (100), dopamine and somatostatin receptors (101), D2/D3Rs (102), and differing subtypes of muscarinic receptors (103). Thus, it is clear that heterodimerization can modulate GPCR responsiveness independently of receptor trafficking.

There are also examples in which heterologous coexpression of two GPCRs leads to agonist-elicited endocytosis profiles different from that of either receptor when expressed alone, presumably owing to heterooligomerization; these studies have been used to provide a functional marker for heterooligomerization in heterologous cells (104–106).

THE ROLE OF CHAPERONES IN GPCR DELIVERY TO THE CELL SURFACE

Molecular chaperones are implicated in cellular quality control owing to their ability to recognize misfolded proteins, presumably by the exposure of usually hidden hydrophobic domains upon protein misfolding. Chaperones also have been implicated in protein targeting and degradation as part of the ER quality control system. Some GPCR-interacting proteins demonstrated to behave as chaperones during GPCR processing are described below.

NINA A The existence of chaperones that support GPCR trafficking to the cell surface was first appreciated for GPCRs that mediate sensory responses. For example, Nina A (*n*either *i*nactivation *n*or *a*fterpotential A) was discovered to be essential for the cell surface expression of rhodopsin 1 (Rh 1) in a screen of *Drosophila melanogaster* defective in visual response to light (107, 108). Nina A is a 237–amino acid photoreceptor-specific integral membrane glycoprotein that shares 40% sequence identity with cyclophilin, a cyclosporin A–binding protein. The cyclophilin protein family is composed of cytosolic proteins that possess peptidyl-prolyl *cis-trans* isomerase activity, which results in *cis-trans* isomerization of the peptide bond between a proline residue and its amino terminal neighbor. *Nina a* mutant flies demonstrated a tenfold reduction of Rh 1 levels in the R1–R6 photoreceptor cells and a reduced level of binding activity for cyclosporin A (CsA) (107). Immaturely glycosylated Rh 1 accumulates in the ER of photoreceptor cells in flies lacking Nina A, indicating that Nina A is required for cell surface expression of Rh 1 (109). More recent studies have implicated the last six amino acids of Nina A in its interaction with Rh 1 (110). Nina A interacts specifically with Rh 1 (and not other *Drosophila* rhodopsins) and is stoichiometrically required for

Rh 1 biogenesis, suggesting that the effectiveness of Nina A in facilitating rhodopsin trafficking to its target membrane is due to its chaperone-like, rather than catalytic, function (110).

ODR-4 The role ODR-4 plays in facilitating the surface delivery of the olfactory receptors ODR10 to cilia in *Caenorhabditis elegans* chemosensory neurons provides an additional example of a chaperone protein enabling a sensory GPCR to reach the cellular surface (111). Although the odorant receptors efficiently reach the cellular surface in specialized olfactory neurons, these receptors are trapped intracellularly when expressed in heterologous cell systems. Dwyer and colleagues employed a positional mapping and transformation rescue strategy in mutant *C. elegans* strains defective in a specific odorant chemotaxis pathway to isolate and clone ODR-4, a novel 445 amino acid (111). ODR-4 is a type II membrane protein with an extreme carboxyl-terminal transmembrane domain. ODR-4 is similar to syntaxin, a protein known to be involved in vesicle transport or targeting (111).

To examine odorant receptor trafficking, perinatal rat olfactory sensory neurons (OSN) were conditionally immortalized by a temperature-sensitive mutant of the oncogene large T antigen, and named *o*lfactory-*d*erived *o*dorant *r*eceptor *a*ctivatable cells (*odora* cells*)*. Under control (undifferentiated) conditions, *odora* cells are phenotypically similar to the OSN progenitor, the globose basal cell. After differentiation, *odora* cells more closely resemble OSNs and have been shown to efficiently target odorant receptors to the plasma membrane (112). Using the *odora* cell line as a model system for OSN, Gimelbrant et al. (113) observed that in undifferentiated cells, odorant receptors were trapped intracellularly at the level of the Golgi, whereas in heterologous cell systems, odorant receptors are retained within the ER. The difference in retention sites of the receptor (i.e., Golgi versus ER) suggests a two-step model for the regulation of olfactory receptor trafficking, with one stage at the level of the ER, with ODR-4 serving a critical role, and a second stage at the level of the Golgi. Another olfactory GPCR, U131, is assisted in its expression at the surface by coexpression with ODR-4 in heterologous cells. In contrast, U131 is retained within the ER when expressed alone in Chinese hamster ovary cells, implicating ODR-4 as a factor in exit of olfactory receptors from the ER (113).

RAMPs Receptor activity modifying proteins (RAMPs) are type I transmembrane proteins expressed in numerous tissues and cell types (Sexton). RAMP1 initially was identified in an effort to clone the receptor for calcitonin gene-related peptide (CGRP) (114). Using an expression cloning strategy in *Xenopus* oocytes, McLatchie et al. identified a single cDNA that encoded a protein that permitted response to CGRP, leading to accumulation of cAMP and subsequent activation of the cAMP-activated chloride channel, CFTR. However, the predicted topography of the protein encoded by this cDNA was not that which would be expected for a seven transmembrane–spanning receptor, but rather that of a single transmembrane–spanning protein with a large N-terminal extracellular region and a short intracellular domain. To date, three RAMP isoforms have been identified

through database analysis and have been named RAMP1, RAMP2, and RAMP3. All share a similar basic domain structure, with 56% sequence similarity but only 31% amino acid sequence identity (114). RAMP1 has been demonstrated to foster transport of the calcitonin receptor-like receptor (CRLR) to the plasma membrane and then remain associated with the CRLR to function as a terminally glycosylated CGRP receptor. RAMP2/3 transport the CRLR to the cell surface and contribute to CRLR functioning as an adrenomedullin (AM) receptor (114).

The discovery of the RAMP accessory proteins controlling the transport, glycosylation state, and binding specificity of the CRLR established a new paradigm for the determination of a GPCR phenotype; the seven transmembrane–spanning CRLR as a malleable template that, in combination with a select RAMP molecule, exhibits selectivity among the family of related calcitonin gene-related neuropeptides (114). Hilairet et al. determined that it is the nature of the protein-protein interactions between CRLR and the various RAMPs, and not the glycosylation state of the CRLR, that determines ligand binding specificity of the CRLR-RAMP complexes (115). All three RAMPs promote maturation of the CRLR to a terminally glycosylated state in heterologous cells (RAMP1 to a greater extent than the other two). Construction of chimeric RAMP molecules leads to receptor specificity data that corroborate the interpretation that the CRLR-RAMP complex determines ligand binding; in fact, it is the extracellular domain of the RAMP proteins that determines ligand selectivity of the CRLR. Crosslinking studies reveal that [^{125}I]-CGRP and [^{125}I]-AM are incorporated into the RAMPs, indicating that RAMPs are in close proximity to the ligand binding pocket of the RAMP-CRLR complex. These data suggest that the RAMPs contribute directly to the ligand binding pocket and can modulate ligand binding.

Once the CRLR-RAMP1 receptor has achieved plasma membrane localization, CRLR, and not RAMP1, is subject to phosphorylation following CGRP stimulation (115a). In response to agonist, the CGRP receptor undergoes dynamin- and β-arrestin-dependent promoted internalization and remains stably associated with β-arrestin, similar to many GPCRs (see below).

Christopoulos et al. (116) explored whether other class II GPCRs could interact with the RAMPs. Among the class II GPCRs investigated, the vasoactive intestinal polypeptide/pituitary adenylate cyclase–activating peptide receptor (VPAC1) interacts with all three RAMPs. In addition, the glucagon receptor interacts with RAMP2; the parathyroid hormone (PTH1) receptor binds both RAMP2 and RAMP3. The possibility that RAMPs may interact with other GPCRs in addition to the CRLR provides a new source of receptor diversity for the investigation of GPCRs (116). In all cases, these interactions lead to both an altered cellular distribution and function of these GPCRs. For example, agonist-evoked PI hydrolysis is significantly enhanced when the VPAC1 receptor is expressed in the presence of RAMP2; thus, RAMP molecules not only appear to affect receptor localization but also may enhance signaling, either by increasing the concentration of receptors available at the surface or perhaps by direct modulation of receptor-effector coupling efficiency.

RCP Another protein, called receptor component protein (RCP), creates a ternary complex with RAMP1 or RAMP2 and CRLR. RCP was originally identified in an expression cloning screen to identify the cDNA encoding the CGRP receptor (117). RCP is a 148–amino acid, peripheral membrane protein attached to the endofacial membrane surface via ionic interactions. RCP appears to facilitate signal transduction by CGRP and AM (118, 119). Similar to the RAMPs, Dickerson and colleagues (119) speculate that the enhancement of signaling mediated by RCP may result either from enhanced coupling between the CGRP-receptor and the signal transduction machinery or by organizing the CRLR at the plasma membrane into a signaling microenvironment, such as into caveolae or lipid rafts. Thus, in the case of the CRLR, the functional receptor responding to either CGRP or AM has expanded to include not only a seven transmembrane–spanning protein (CRLR) and a chaperone (RAMP) but also a signal transduction accessory protein (RCP) as well.

CALNEXIN Calnexin has been shown to assist in the folding of a number of GPCRs. Calnexin binds to monoglucosylated N-linked carbohydrate chains and peptide determinants on folding intermediates of target proteins, such as the precursor form (i.e., Endo H-sensitive form) of the V2R. Many of the V2R alleles associated with X-linked NDI are retained intracellularly. Morello et al. observed that the NDI allele R337X V2R, encoding truncation of the cytosolic cytoplasmic tail of the receptor, is retained in the ER and colocalizes with the ER marker protein calreticulin. Calnexin actually appears to interact more robustly with mutant alleles of the V2R associated with NDI than with the precursor form of the wild-type receptor. These findings suggest that calnexin also plays a role in proper folding and maturation of the V2R, thereby promoting appropriate trafficking to the cell surface.

Calnexin has been implicated in a similar role for the lutropin/choriogonadotropin receptor (LHR), follicle-stimulating hormone receptor (FSHR), and thyrotropin-stimulating hormone receptor (TSHR) (120), receptors that also interact with two other known ER folding/chaperone proteins, BiP and GRP94 (121). In the case of the TSHR, calnexin and calreticulin stabilize the receptor during initial synthesis, whereas BiP overexpression leads to increased receptor degradation.

RANBP2 A search for mammalian cyclophilins related to *Drosophila* NinaA by probing a bovine retinal cDNA library with a NinaA cDNA probe led to the cloning of two alternatively spliced cDNAs encoding proteins named cyclophilin type I and II (122). The predicted amino acid sequence of the cyclophilin type II protein was found to be identical to RanBP2, a molecule known to bind the GTPase Ran. RanBP2 is a chaperone for vertebrate red/green opsin and contains two key domains (the Ran-binding domain 4 and the cyclophilin domain) that act in concert to create its chaperone function (123). The cyclophilin domain does not bind directly to opsin, but stabilizes the interaction between red-green opsin and the Ran-binding domain 4. Like NinaA, which specifically interacts with one isoform of rhodopsin (Rh1), RanBP2 acts as a specific chaperone for red/green opsin in the mammalian retina.

HSJ1b Mammalian rhodopsin recently has been shown to also interact with the DnaJ/Hsp40 cochaperone protein HSJ1b. Proteins in this family of cochaperones are characterized by a highly conserved 70–amino acid J-domain that interacts with Hsp70. HSJ1b is preferentially expressed in neurons, is geranylgeranylated, and localizes to the cytoplasmic face of the ER. Although coexpression of HSJ1b with rhodopsin in heterologous cells leads to accumulation of both proteins in the ER and in aggresomes, no aggregation of these proteins into aggresomes or in the ER is observed in vivo. These findings implicate the existence of yet another protein in retinal cells that enables rhodopsin to achieve surface localization without HSJ1b-driven aggregation and ER accumulation (124).

gC1q-R A yeast two-hybrid search for novel proteins that interact with the cytoplasmic carboxyl-terminal cytoplasmic tail of α_{1B}-AR led to the identification of gC1q-R (receptor for globular heads of C1q) as a potential α_{1B}-AR-binding partner (125). gC1q-R is a multifunctional protein that was originally identified as a complement regulatory factor (126, 127). Coimmunoprecipitation assays in COS7 cells confirmed the interaction between full-length gC1q-R and the α_{1B}-AR in intact cells. Colocalization of α_{1B}-AR and gC1q-R in cells using fluorescence confocal laser scanning microscopy corroborates these findings (128).

The C terminus of the α_{1B}-AR contains a series of sequence motifs (see above) common to GPCRs (and described in Motifs Implicated in GPCR Trafficking to the Cell Surface, above): an NPXXY motif thought to play a role in receptor trafficking, a putative acidic/dihydrophobic motif implicated in cell surface delivery, and an arginine-rich region. Binding analysis in the yeast two-hybrid assay system revealed that the arginine-rich region of α_{1B}-AR (amino acids 371–378) facilitates interaction with gC1q-R, whereas the NPXXY motif and the acidic/dihydrophobic domain do not (125). When individually expressed in heterologous cells, the α_{1B}-AR exhibits a distribution pattern characteristic of a plasma-membrane associated protein, whereas gC1q-R localization is predominantly cytoplasmic (125). Interestingly, when the two proteins are coexpressed, the α_{1B}-AR shifts from a cell surface to a cytoplasmic intracellular distribution, and functional α_{1B}-AR declines. This redistribution is not detected for an α_{1B}-AR truncation mutant lacking the arginine-rich motif. These findings emphasize the potential importance of the interaction between α_{1B}-AR and gC1q-R in dictating maturation and expression of the α_{1B}-AR as a cell surface protein.

MOLECULES THAT RETAIN GPCRS AT THE CELL SURFACE

As stated above, efficient signal transduction requires a receptor to be in the proper location at the precise time to interact with its endogenous ligands. As such, a number of GPCR-interacting proteins other than those involved in signaling (e.g., G proteins, RGS proteins, effectors) have been identified that have been shown to

delay the turnover of the receptor at the cell surface; these are discussed below. Perhaps future studies will reveal the role of these interacting proteins in scaffolding of signaling complexes, as for A-kinase anchoring proteins (128a) or proteins with PDZ domains (128b). One such protein is arrestin. However, because arrestin interaction with GPCRs is fostered significantly by agonist activation and plays an important role in agonist-accelerated GPCR endocytosis, the impact of GPCR-arrestin interactions is addressed in Agonist-Evoked Receptor Endocytosis (below).

The Demonstrated Role of Some GPCR-Interacting Proteins in Stabilizing GPCRs at the Cell Surface

Many GPCR-interacting proteins have been demonstrated, by biochemical or morphological strategies, to stabilize GPCR expression at the cell surface. Several of these are described in more detail below.

SPINOPHILIN Both D2Rs (129) and α_2ARs (130) interact with spinophilin (Neurabin II), a ubiquitously expressed 120-kDa multidomain protein. Spinophilin initially was identified as a protein phosphatase 1 (PP1)-binding protein localized in dendritic spines, hence the name spinophilin (131), and an F-actin-binding protein underlying the E-cadherin-enriched basolateral surface of polarized renal epithelial cells (132). Spinophilin also contains three coiled-coil domains at its C terminus, which mediate homomultimerization in vitro (132) and provide the capability of forming multiprotein complexes in cells. The D2R "short" and "long" isoforms and all three α_2-AR subtypes interact with spinophilin via their third intracellular (3i) loops by binding to a region of spinophilin sequence that links the F-actin binding and PP1 regulatory domains (129, 130). Smith and colleagues (129) additionally showed that both the D2R and PP1 could interact with GST-spinophilin at the same time, supporting the notion that spinophilin may be a scaffolding protein that links signaling proteins and their regulators to microdomains at the cell surface.

Three lines of evidence caused us to postulate that α_2-AR-spinophilin interactions might stabilize the α_2-AR on the basolateral surface of polarized cells: (*a*) The α_2-AR subtypes interact with spinophilin via their 3i loops (129, 130), (*b*) the α_{2A}-AR is retained at the basolateral surface of polarized epithelial cells via its 3i loop (12, 133), and (*c*) spinophilin is enriched beneath the basolateral surface to which the α_{2A}AR is tethered (132). We observed that redirection of the GPCR-interacting domain of spinophilin to the apical surface of polarized MDCK II cells by fusion with p75 (a subunit of the nerve growth factor receptor) leads to delay of turnover of the α_{2B}-AR subtype on that surface (25) following its random delivery there (13). Second, α_{2B}-AR transduced into mouse embryo fibroblasts (MEFs) derived from wild-type versus spinophilin knockout ($Sp^{-/-}$) mice demonstrate a much faster agonist-induced internalization in $Sp^{-/-}$ MEFs than in $Sp^{+/+}$ MEFs (25). These data are consistent with the interpretation that spinophilin stabilizes GPCRs at the cell surface.

HOMER Homer [now designated as Homer-1a or Vesl (*V*ASP/*E*na-related gene upregulated during *s*eizure and *L*TP)] is a 186–amino acid protein containing a PSD95/*Drosphila* discs large/*Zona occludens* (PDZ)-like domain in its N-terminal region. Homer-1a was originally identified in a screen to reveal molecules involved in long-term plasticity (134). It is now known that three genes encode a Homer protein family; all members share a conserved 120–amino acid N-terminal region homologous to the EVH1/WH1 (Ena/VASP homology 1/WASP homology 1) domain, implicated in the control of actin filament dynamics (135). Homer-1a is an immediate early gene whose expression is upregulated in the hippocampus following excitatory synaptic activity. Unlike Homer-1a, Homer-2 and Homer-3 are both constitutively expressed and have a carboxyl-terminal coiled-coil motif (136). Homer-1a competes via its N-terminal sequences with Homer-2/3 for binding to mGluR, and thus can function as a dominant-negative structure for the signaling-associated proteins, Homer 2/3 (see below), delaying and reducing mGluR-induced Ca^{2+} transients in cerebellar Purkinje cells (137).

Genetic and biochemical strategies (134) have revealed an interaction between Homer and the C-terminal tail of the metabotropic glutamate receptor, mGluR5. Because the C-terminal tail of mGluR1α is 67% identical to the carboxyl terminus of the mGluR5 over its last 55 amino acids, and both receptors terminate with four PDZ-domain-binding amino acids (SSTL/SSSL respectively), it is not surprising that mGluR5 and mGluR1α bind a GST-Homer fusion protein. Deletion of the four-amino acid PDZ-domain-binding motif from mGluR5 reduces binding to Homer, and deletion of the last ten amino acids of this mGluR subtype eliminates binding. Although mGluR2 and mGluR3 also share a PDZ-domain-binding motif in their last four amino acids, these receptors diverge significantly from mGluR1α and mGluR5 in C-terminal sequences outside of this region. Neither mGluR2 nor mGluR3 bind Homer, indicating that the C-terminal four amino acids are necessary but not sufficient for receptor binding to Homer. The mGluR4 shows little sequence homology with any of the other members of this family in these sequence regions and also does not bind Homer.

Homer proteins play a documented role in trafficking of group I mGluRs to the cell surface. Roche et al. (138) presented evidence that Homer-1b causes retention of group I mGluRs in the ER, while Homer-1a allows the mature receptor to be inserted into the plasma membrane. However, once the mGluR1α is inserted at the cell surface, Homer-1a does not remain stably associated with the receptor. In contrast, cell surface group I mGluR appear to interact with Homer-1c. In cells overexpressing Homer-1c, mGluR1α redistribute into large clusters and manifest a longer surface half-life than in the absence of overexpressed Homer-1c; glutamate activation of IP_3 production also is increased when Homer-1c is coexpressed with mGluR1α. Transient transfection of primary cultures of rat cortical neurons with Homer-1c and mGluR1α leads to increased receptor localization in the dendrites of these neurons (139).

A similar scenario is observed in cultured cerebellar granule cells (140). Recombinant Homer-1a alone does not affect cell surface expression, but coexpression

with Homer-1b results in intracellular retention and clustering at synaptic sites. Similarly, in cells transfected with both Homer-1a and -1b, excitation-induced expression of native Homer-1a reversed the intracellular retention of mGluR5 and allowed for receptor trafficking to the synaptic membranes.

Taken together, the data suggest an array of interactions of mGluR with Homer isoforms during a receptor lifetime (134–141). Nascent group I mGluRs accumulate in the ER associated with Homer-1b. Homer-1a protein, induced by synaptic activity, competes for receptor-Homer-1b interactions in the ER to facilitate mGluR trafficking to the cell surface. There, they can engage in stable interactions with Homer-1c or with other mGluR-interacting proteins. Homer also may function to negatively regulate the constitutive (ligand-independent) activity of the mGluR.

Deletion analysis has identified a novel proline-rich Homer ligand motif (PPSPF) in group I mGluRs (mGluR5 and mGluR1α) 50 amino acids upstream from the receptor C terminus (137). The same motif exists in other Ca^{2+} mobilizing receptors, such as the IP_3 receptor (IP_3R) and the ryanodine receptor (RYR). Interestingly, Homer proteins have been shown to immunoprecipitate both IP_3R and mGluR1α from cerebellum homogenates (137).

Homer interacts not only with GPCRs but also with Shank family proteins, linking Homer to GKAP, PSD-95, and NMDA receptor-containing complexes. It has been postulated that these interactions may facilitate cross-talk between Homer-stabilized metabotropic and ionotropic receptors at glutamatergic synapses (142).

ACTIN BINDING PROTEIN-280/FILAMIN A Actin binding protein-280 (ABP-280), also known as filamin A, is an abundant cytoplasmic protein that contains an actin-binding domain at its N terminus followed by 24 tandem immune globulin-like repeats of approximately 96 amino acids each (143), two hinge regions, a dimerization domain at its C terminus, and an actin binding domain at its N terminus. This molecule has been shown to interact with several GPCRs. For example, ABP-280 interacts with the 3i loop of the D2 short and long isoforms of the D2R and with the D3R, but not with the D4R orD1R3i loops. Interestingly, although D2R exhibits similar affinities for agonists and antagonists in ABP-280-deficient M_2 human myeloma cells and in M_2 cells heterologously expressing ABP-280, the ability of the D2R to inhibit forskolin-stimulated cAMP accumulation is dramatically reduced in cells lacking ABP-280.

A protein kinase C (PKC) phosphorylation site at serine 358 within the D2R 3i loop is an important regulatory site for the ABP-280 interaction with this receptor. Mutation of this serine to aspartic acid, intended to mimic the serine-phosphorylated state, significantly reduces both ABP-280 binding to the D2R and agonist potency in receptor-mediated inhibition of adenylyl cyclase, an observation that is mimicked by direct activation of PKC with PMA (4β-phorbol-12-myristate-13-acetate) in these cells. One interpretation of these data is that PKC phosphorylation of the 3i loop of the D2R disrupts ABP-280 binding and is followed by a loss of receptor-effector coupling.

ABP-280/filamin A also interacts with the Ca^{2+}-sensing receptor (CaR) (144). CaR activation of MAP kinase is not observed in M_2 melanoma cells lacking ABP-280; however, this activity is restored upon exogenous expression of ABP-280 in these cells. Thus, ABP-280 appears critical for signaling of D2R and of CaR in human myeloma cells.

ABP-280 also fosters D2R clustering at the plasma membrane. Interestingly, the D1R, which does not detectably interact with ABP-280, also clusters along the plasma membrane in all cells assayed regardless of ABP-280 expression. These data suggest that a different interacting partner (in addition to, or distinct from ABP-280) may be responsible for microcompartmentalization of this receptor at the cell surface.

ABP-280 also appears to modulate GPCR localization after the receptor has reached the cell surface. Upon finding that the calcitonin receptor (CTR) undergoes tonic internalization and that ligand stimulation leads to both increased internalization and receptor recycling to the cell surface, Seck and colleagues postulated that a cellular protein associated with the C-terminal tail of the receptor was necessary for targeting the CTR to the recycling compartment (145). Screening of an osteoclast-like cell yeast two-hybrid library with the CTR C-terminal tail as bait revealed an interaction with the carboxyl-terminal region of ABP-280 that contains the 19–24 immunoglobulin-like repeats and the second hinge region. The CTR and endogenous ABP-280 were shown to interact in mammalian cells via coimmunoprecipitation, but this interaction did not appear to be regulated by calcitonin stimulation. The findings for the D2R and CaR noted above suggest that association of CTR with ABP-280 is not necessary for ligand-induced generation of cAMP, increases in intracellular calcium, or ERK phosphorylation, as these signaling events are indistinguishable in M_2 cells (no endogenous ABP-280) versus A7 cells (M2 stably expressing ABP-280). However, both confocal microscopy and fluorescence-activated cell sorter (FACS) analysis suggested that there was less surface expression of CTR in the M_2 cells than in A7 cells expressing ABP-280. This difference was due to increased receptor degradation in the ABP-280-deficient M2 cells. It appears that the presence of ABP-280 is critical for CTR recycling from an endocytic compartment back to the cell surface (145).

PROTEIN 4.1N Protein 4.1N is a member of the protein 4.1 family of cytoskeletal-associated proteins that is specifically enriched in neurons. It is presumed that it links the spectrin-actin cytoskeleton to the cell membrane, and thus may play a role in modulation of the stability and plasticity of the neuronal plasma membrane (146). Protein 4.1N previously was shown to interact with the GluR1 subunit of the ionotropic AMPA receptor and to colocalize with AMPA receptors at excitatory synapses (147). Recently, this protein was identified as a GPCR (D2R/D3R)-interacting protein (148).

The most C-terminal region of protein 4.1N is necessary and sufficient to mediate the interaction with the D2/D3R; this region of protein 4.1N also displays the highest degree of homology among the four different protein 4.1 isoforms,

suggesting that interaction with protein 4.1 family members may be a mechanism for linking D2 and D3Rs to the cytoskeleton (148).

Consistent with a role for 4.1N proteins in GPCR tethering or stabilization at the cell surface are the findings that a protein 4.1N truncation mutant, which competes for dopamine receptor binding but does not bind to the plasma membrane or the cytoskeleton, is unable to stabilize D2Rs or D3Rs at the cell surface, leading to a ~50% reduction in cell surface molecules. Thus, appropriate targeting or stabilization of the D2/D3R at the cell surface may be dependent upon its interaction with protein 4.1N.

Although ABP-280 (see above) interacts with the same region of the D2 dopamine 3i loop as protein 4.1N (149), extant data suggest that ABP-280 and protein 4.1N may bind simultaneously to the D2R. This interpretation is based on the lack of effect of overexpression of protein 4.1N on the amount of ABP-280 coimmunoisolated with the D2R.

MUSKELIN Muskelin is a widely expressed intracellular protein containing six dispersed C-terminal motifs that exhibit homology to the tandem repeats first identified in the *Drosophila* kelch ORF1 protein (150). Muskelin was discovered during expression cloning of thrombospondin-1 response molecules in COS cells. The so-called kelch motifs in muskelin are thought to be involved in actin binding (151).

Muskelin interacts with the carboxyl tail of the EP3α receptor, but not with the carboxyl tail of the EP3β or EP3γ splice variants, which differ in their C-terminal sequences (152). The EP3 receptor binds prostaglandins and is linked to the inhibition of adenylyl cyclase and activation of the small GTPase Rho. Alternative splicing leads to the expression of three isoforms of the EP3 receptor: EP3α, EP3β, and EP3γ. Each isoform displays a unique carboxyl-terminal tail sequence that may provide the basis for previously observed differences in agonist-dependent desensitization, agonist-independent G_i activity, and membrane targeting (152). Both EP3α receptors and muskelin are localized to the plasma membrane following transient coexpression, although a diffuse cytoplasmic pool of muskelin also can be detected (152). This may be the result of inhibition of agonist-stimulated EP3αR internalization by coexpression of muskelin. The EP3α isoform in HEK293 cells facilitates EP3-mediated inhibition of EP2 receptor (G_s coupled)-stimulated cAMP formation (152). Muskelin thus may play a role in anchoring or subcellular targeting of the EP3α receptor to the plasma membrane, hence, modulating EP3αR signaling and/or receptor lifecycle.

PSD-95 PSD-95 (postsynaptic density-95) has been shown to interact with β_1-ARs via a C-terminal sequence that represents a PDZ-interacting domain (153). Interaction of the β_1-AR with PSD-95 appears to stabilize the receptor on the cell surface, as mutations in the β_1-AR C terminus disrupt colocalization with PSD-95. By contrast, overexpression of PSD-95 decreases β_1-AR internalization, although this consequence is not paralleled by changes in agonist-elicited β_1-AR

activation of cAMP production or subsequent desensitization. Although PSD-95 interaction with the β_1-AR does not directly regulate its signaling, it appears that β_1-AR-PSD-95 complexes can facilitate interaction with *N*-methyl-D-aspartate (NMDA) receptors in target cells (153), thus providing the molecular bases for a microcompartmentation that could mediate the known β_1-AR regulation of NMDA currents in neurons (154–156).

Other GPCR-Interacting Proteins for which a Possible Role in Tethering to the Cell Surface Has Not Been Established

Not all GPCR-interacting proteins have established functions. Those for which receptor tethering to the cell surface has not been documented are summarized below.

CALMODULIN The G_q-coupled mGluR5 was the first GPCR reported to interact directly in a Ca^{2+}-dependent manner in vitro with calmodulin (CaM) (157). CaM is a ubiquitous 17 kDa Ca^{2+} binding protein that modulates the activity of a number of biochemical and electrical signaling pathways (158). Minakami et al. characterized two distinct CaM binding sites on the mGluR5, each of which can be phosphorylated by PKC and each possessing different affinities for CaM (157). The G_i/G_o-coupled mGluR7 also interacts with CaM in a Ca^{2+}-dependent manner (159). As for mGluR5, the CaM binding domain for mGluR7 undergoes phosphorylation by PKC; phosphorylation by PKC and CaM binding are mutually antagonistic (157). Further, CaM and G protein $\beta\gamma$ subunit binding also occurs in a mutually exclusive manner (160). Although CaM binding is not necessary for G protein activation, it is required for $G_{\beta\gamma}$ subunit dissociation from the carboxyl tail of mGluRs to mediate $\beta\gamma$-dependent glutamatergic signaling pathways (160). Although it is apparent that CaM is involved in masking phosphorylation sites and facilitation of G protein signaling, whether or not this results from CaM-regulated changes in cell surface GPCR stabilization has not been established.

A sequence-based motif search uncovered yet another GPCR likely to bind CaM, the μ-opioid receptor (161). Electrophoretic mobility shifts of CaM on nondenaturing gels revealed that the C-terminal 3i loop of the μ-opioid receptor (OP_1 and OP_3) binds CaM in a calcium-dependent manner. Peptides derived from the 3i loop of OP_3 bound strongly to CaM and suppressed interaction between solubilized OP_3 and CaM, indicating that a direct interaction may be occurring between CaM and OP_3 receptors (161). Furthermore, Wang and colleagues found that CaM regulates basal and agonist-stimulated G protein coupling of OP_3 (162).These authors conclude that by directly competing with G proteins for binding to OP_3, CaM may serve as an independent second messenger that is activated following receptor stimulation by agonist (161, 162). Subsequent studies by this same group investigated the functional effect of three different previously identified single nucleotide polymorphisms (SNPs) that map to the μ-opioid receptor 3i loop. Wang and colleagues found that each of these polymorphisms significantly reduced basal

G protein coupling and/or resulted in deficient CaM binding, suggesting that G protein coupling and CaM binding domains partially overlap (163). A more thorough understanding of the functional impact of these polymorphisms on signaling activity and receptor regulation will be important owing to their potential ability to affect the response to opioid analgesics of individuals harboring these different alleles.

A CaM-binding motif (defined by several hydrophobic residues interspersed with a number of positively charged residues, arginine and lysine) in a stretch of 14 amino acids (164) also has been identified in the 3i loop of the D2R within the same region of the 3i loop (amino-terminal) responsible for activation of $G_{i/o}$ (165). A 3i loop peptide encompassing this domain binds CaM in a Ca^{2+}-dependent manner in cross-linking studies; the affinity of the 3i loop peptides for CaM is estimated to be $\sim$ 80 nM (165). Additional studies have revealed that Ca^{2+}/CaM suppresses activation of purified $G_{i/o}$ by the 3i loop peptide (165). Rhoads & Friedberg (164) also found that D2 receptor-stimulated GTP$\gamma^{[35]}$S binding is inhibited by the presence of Ca^{2+}/CaM. Despite the apparent competition of CaM and G proteins for interaction with GPCR in functional studies, Bofill-Cardona and colleagues observed that immobilization of $G_{\alpha i}$ on CaM-agarose resin requires the presence of the D2R 3i loop peptide, indicating that the receptor peptide was capable of simultaneous interaction with both CaM and $G_{\alpha i}$. These findings would be consistent with the existence of a ternary complex between GPCR, G protein, and CaM; CaM-mediated inhibition of D2R signaling would occur by blocking receptor-catalyzed G-protein turnover.

It is of interest to note that the D2R, like the mGluR7 and the μ-opioid receptors, is coupled to G proteins of the $G_{i/o}$-subfamily and that all three receptors are under the regulatory control of CaM, a molecule for which they have similar binding affinities (i.e., 60–100 nM) (159–161, 165). Nevertheless, CaM appears capable of exerting opposing functions on these receptors; CaM enhances mGluR7-mediated effector function (i.e., Ca^{2+} channel inhibition) and suppresses μ-opioid and D2R-mediated signaling. Consequently, CaM represents an accessory signaling molecule that can coordinate signaling and affect distinct cellular responses by targeting and discriminating between discrete cytoplasmic domains within even closely related GPCRs.

CaM was most recently identified as a 5-HT_{2C} C-terminal tail interacting protein by Becamel and colleagues through the use of an innovative proteomic approach to screen for novel GPCR-interacting protein partners (166). This group used a GST-fusion protein containing the entire 90-amino acid 5-HT_{2C} C-tail to specifically isolate interacting proteins from mouse whole-brain extract. Isolated proteins were resolved by two-dimensional gel electrophoresis and unequivocally identified by MALDI-TOF mass spectrometry. CaM was among 15 different interacting proteins identified in this particular screen, and unlike several of the other proteins identified, it bound the 5-HT_{2C} independent of the C-terminal PDZ binding domain (166). The functional consequence of CaM binding to the 5-HT_{2C} receptor remains to be determined.

CALCYON Calcyon is a 24-kDa, 217-residue protein containing a predicted single transmembrane–spanning segment. This protein not only interacts with D1Rs in two-hybrid screens but also is localized to similar brain regions as the D1R, such as the dendritic spines of D1R-expressing pyramidal cells in the prefrontal cortex. Lezcano and colleagues (167) have named calcyon for its apparent role in dopamine receptor signaling, where its coexpression with D1R leads to an increase in intracellular Ca^{2+} release (i.e., turning calcium on) in response to D1R agonist owing to enhanced D1R coupling, presumably to G_q. In the absence of calcyon overexpression, the D1R is coupled predominantly to G_s and stimulated cAMP production.

14-3-3 PROTEINS The 14-3-3 proteins (168) interact with a number of signaling proteins and have been interpreted to act as a signaling scaffold (169) or as a molecular "buffer," or interfering molecule (170). The ζ isoform of 14-3-3 was demonstrated to interact with all three α_2-AR subtypes via their 3i loops (171); in addition, two isoforms of 14-3-3, η and ζ, interact with the C-terminal tail of the $GABA_BR1$ (172). This interaction of 14-3-3 is specific to $GABA_BR1$ and does not occur with $GABA_BR2$ either in yeast two-hybrid assays or in GST-pull down experiments with mammalian preparations. Although $GABA_BR1$ colocalizes with 14-3-3 in immunostaining in cultured hippocampal neurons and following fractionation of rat brain, coimmunoprecipitation of these proteins from native tissues cannot be detected (172), consistent with earlier findings for the α_2AR (171). These data suggest that GPCR-14-3-3 protein interactions are of relatively low affinity (173). The domain of $GABA_BR$ involved in binding of 14-3-3 ζ is the C-terminal coiled-coil region of $GABA_BR1$, i.e., the same domain implicated in heterodimer formation with $GABA_BR2$. Binding of a radiolabeled carboxyl-terminal $GABA_BR1$ probe to an immobilized GST fusion protein encoding the $GABA_BR2$ carboxyl-terminal tail is disrupted by 14-3-3ζ, suggesting that 14-3-3 may regulate GABA receptor heterodimer formation in the context of a cell, and by virtue of this interaction, alter delivery of functional $GABA_B$ heterodimeric receptors at the cell surface.

Whether the interaction of 14-3-3 proteins with RGS proteins (9) is functionally interrelated with the interactions of 14-3-3 proteins with GPCRs (171, 172) has not yet been clarified. Nor has it been established whether these interactions play a role in GPCR trafficking, be it receptor delivery to a particular surface compartment or retention in that compartment.

TURNOVER OF GPCRS ON THE CELL SURFACE

The previous sections have addressed receptor delivery to the cell surface (Delivery of Nascent GPCRs to the Cell Surface, above) and retention at that surface (Molecules that Retain GPCRs at the Cell Surface, above). This section addresses GPCR turnover at the cell surface in the agonist-naïve (Impact of Intrinsic

Receptor Stucture on Turnover of GPCRs at the Cell Surface, below) and agonist-evoked (agonist-Evoked Receptor Endocytosis, below) states.

Impact of Intrinsic Receptor Structure on Turnover of GPCRs at the Cell Surface

Subtle changes in the amino acid sequence of GPCRs, which may or may not profoundly alter receptor structure, may nonetheless alter their surface turnover. For example, intentional mutagenesis of the α_{1B}-adrenergic receptor (174), β_2-adrenergic receptor (175), or α_2-adrenergic receptor (176) to evoke constitutive, agonist-independent activation results in GPCRs with accelerated turnover at the cell surface (177, 178). These rapidly turning over GPCRs also are less stable in membranes and in detergent solution but can be stabilized by agonist or antagonist occupancy of the receptor (175, 177, 178). GPCR structures that manifest agonist-independent activation are assumed to exhibit structural instability owing to mimicry of the GPCR agonist-activation switch conformation (177, 178).

The impact of site-specific changes in amino acid sequence on agonist-independent (constitutive) activity of GPCRs has been most extensively studied for the α_{1B}-adrenergic receptor (α_{1B}-AR). Here, a single site was substituted by all 23 amino acids, and a graded continuum of agonist-independent receptor activation was observed (179). These findings imply that native receptor structures also represent a continuum of agonist-independent activity. Sensory receptors, e.g., rhodopsin, are functionally silent in the absence of ligand (e.g., light in the case of rhodopsin), a necessary prerequisite for the extraordinary signal:noise ratio required in an appropriately responding sensory system. For rhodopsin, in particular, this absent "basal" activity of opsin may be due to the constitutive presence of a covalently bound inverse agonist, the *cis*-retinal. In contrast, various GPCR subtypes observed in multiple target tissues often possess different degrees of agonist-independent activation, even when examined in native target cells, i.e., at normal physiological receptor density. (It has often been demonstrated that receptor overexpression is paralleled by an increase in agonist-independent receptor activation (179, 179a). The assumption is that the variable amount of agonist-independent GPCR activation in target cells allows basal levels of cellular second messengers controlled by a given GPCR to be generated as appropriate for homeostasis of the target cell function.

Serotonin receptors represent an example of GPCR where differing receptor subtypes possess differing degrees of constitutive activity (180). Furthermore, RNA editing of $5HT_{2C}$ receptors (181) leads to edited isoforms with enhanced constitutive activity. By analogy with α_1-, β_1-, and α_2-ARs, these constitutively active receptor variants may possess accelerated receptor turnover, although this has not been investigated directly, which would alter steady-state receptor density, signal duration, and overall cellular sensitivity.

A number of alleles of GPCRs that encode constitutively active receptors lead to disease; some of these have been demonstrated to exhibit structural instability

and/or accelerated surface turnover (182). As an example, the R137H V2 vasopressin allele encodes a V2R that constitutively binds arrestin, leading to a receptor that is constitutively internalized and not available for AVP stimulation at the cell surface, leading to X-linked NDI (183).

Perturbations of receptor structure other than those that enhance agonist-independent activity also can destabilize GPCR structure, leading to both instability in detergent solution as well as accelerated surface turnover. For example, the mutant D79N α_{2A}-AR, altered at an Asp residue at a highly conserved position embedded in TM2 of amine-binding GPCRs, results in a destabilized receptor that has perturbed coupling to G proteins, instability in detergent solution, accelerated surface turnover, and significantly (80%) reduced steady-state receptor density in vivo when compared to the wild-type receptor (184). Interestingly, an allele that encodes a V2R responsible for X-linked NDI, the D85N V2R, is in a position analogous to that of the D79N α_2-AR and may possess the same instability, although not yet investigated, which would explain diminished V2R function observed for this mutant receptor (185).

It is likely that SNPs within GPCR coding regions will encode a number of GPCRs with cell surface turnover that differs from the presumed wild-type structure for that receptor and that the differential turnover of these GPCRs will have an impact on the potency of agonists in receptor-mediated signaling, to the extent that cell surface density of a receptor is altered.

Agonist-Evoked Receptor Endocytosis

Agonist-elicited receptor sequestration and endocytosis from the surface of target cells has been implicated in long-term desensitization to receptor signals and has been reviewed extensively elsewhere (186, 187). This pathway of receptor trafficking is only briefly addressed here. However, it should also be noted that GPCR sequestrations and endocytosis may additionally provide a means of propagation of GPCR signals to specific compartments within cells (188, 189).

Although receptor internalization is a common process for GPCRs, different receptors (or different subtypes within the same receptor family) exhibit different properties following agonist stimulation. The intrinsic receptor properties, i.e., the ability of a GPCR to interact with other regulatory proteins such as arrestin, control the rate and extent of agonist-elicited turnover, as well as the fate (recycling versus degradation) after endocytosis. For example, the 3i loop of the α_{2B}-AR subtype has a much higher affinity for arrestin than the α_{2A} subtype (190), and consequently, the α_{2B} subtype undergoes a rapid agonist-evoked sequestration when compared to the α_{2A}-AR subtype (16, 20, 176, 191). Similarly, β_1-AR has a much lower affinity for arrestin than β_2-AR and exhibits resistance to agonist-induced sequestration (192). These two examples underpin the concept that molecules, such as arrestin, can play a critical role in endocytosis of a number of GPCRs; however, as discussed below, some GPCRs can undergo arrestin-independent endocytosis.

At least three agonist-evoked pathways for accelerated removal of GPCR from the cell surface have been described. These pathways have been revealed in cells expressing heterologous GPCR, often at densities that may exceed the capacity of endocytosis machinery or mechanisms that result from interaction with limiting concentrations of relevant regulatory proteins. However, which of these pathways dominates in vivo in native target cells is only now being explored in genetically engineered mice. An example of the importance of reconfirming heterologous expression results in native cells of interest is revealed in the studies of Vogler et al. (193), which demonstrated that a role for RhoA in inhibiting agonist-induced sequestration of M1 and M2 muscarinic receptors implicated by studies in RhoA-overexpressing cells was not corroborated by treatment of cells not overexpressing the small GTPase RhoA with expression of C3 transferase, an inhibitor of RhoA function.

Three principal pathways reported for cell surface turnover of multiple GPCR in heterologous systems include (*a*) arrestin- and dynamin-dependent; (*b*) arrestin-independent, dynamin-dependent endocytosis; and (*c*) arrestin- and dynamin-independent endocystosis (194). GPCRs can internalize via two distinct machineries, i.e., clathrin-coated pits and caveolae (195, 196). A recent report suggests that selection among these machineries may be driven by the particular sites phosphorylated on a GPCR, representing substrate sites for G protein-coupled receptor kinase (GRK) versus PKA (197), with the β_1-AR being the GPCR example. Thus, both GRK- and PKA-mediated phosphorylation of the β_1-AR lead to desensitization of agonist-elicited cAMP accumulation, GRK-catalyzed phosphorylation directs the β_1-AR to clathrin-coated pits, and PKA-catalyzed phosphorylation directs the receptor to caveolae-mediated internalization. This is in contrast to the β_2-AR, where cAMP cannot promote its endocytosis.

ARRESTIN- AND DYNAMIN-DEPENDENT ENDOCYTOSIS The arrestin- and dynamin-dependent agonist-elicited receptor internalization pathway is the paradigmatic model for agonist-evoked GPCR endocytosis and has been most extensively studied for the β_2-AR [reviewed by Claing et al. (194)]. There are four members of the arrestin regulatory family of proteins. Two visual arrestins (rod and cone) are expressed predominantly in the retina where they function in the desensitization of rhodopsin. Two nonvisual arrestins, arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2), are expressed in a wide variety of tissues. Receptor phosphorylation by GRKs enhances binding of arrestins, which serves (*a*) to quench agonist-mediated G protein signaling, and (*b*) to act as a scaffolding intermediate with AP2 and clathrin, stabilizing association with clathrin-coated pits. Endocytosis of these pits requires the GTPase dynamin, which apparently oligomerizes and leads to an extended dynamin helix that promotes extension of the neck of the budding vesicle, leading to vesicle fission (194–96). Here, the receptor is both dephosphorylated and recycled to the cell surface (resensitization) or degraded (downregulation) (197).

Another emerging understanding of the molecular events that attend, and perhaps mediate, arrestin- and dynamin-dependent GPCR endocytosis is the

ubiquitination of both receptor and arrestin (198–200). Ubiquitination of these two proteins is rapidly accelerated by agonist stimulation of β_2-ARs (199, 200) or chemokine receptors (198). Extant data suggest that ubiquitination of the receptor and arrestin have distinct roles, each of which is obligatory for trafficking and degradation of GPCRs. Thus, arrestin ubiquitination is critical for GPCR internalization (198, 199); in fact, the ubiquitination status of arrestin determines the stability of the GPCR-arrestin complex. However, elimination of arrestin ubiquitination does not significantly perturb GPCR degradation postendocytosis. In contrast, mutation of GPCRs, e.g., the β_2-AR, to remove lysine residues critical for receptor ubiquitination leads to ineffective GPCR degradation without attenuating internalization.

Other proteins also are implicated in modulating the arrestin- and dynamin-dependent GPCR endocytosis pathways, including ARF6; its guanine nucleotide exchange factor ARNO; and a GRK-interacting protein, GIT, a GTPase activating protein for ARF (201, 202). Arrestin interacts with both ARNO and the GDP-bound form of ARF6 (202). Overexpression of ARNO increases endocytosis of β_2AR; by contrast, overexpression of GIT1 or ARF6 mutants defective either in GTP binding or in GTP-hydrolysis inhibits agonist-evoked β_2-AR endocytosis (201, 202). A role for Src-mediated tyrosine phosphorylation of dynamin in clathrin-mediated internalization of β_2-AR also has been shown (203) and it is probable that arrestin plays a causal role in this Src-catalyzed tyrosine phosphorylation of dynamin.

That a GPCR internalizes via a pathway that involves both arrestin and dynamin would be manifest by sensitivity to tools that inhibit each of these pathways. For example, mutant arrestin structures have been developed that block binding to AP2 (204) and/or clathrin (205) and serve as dominant-negative modulators of GPCR internalization. Mini-gene constructs expressing the clathrin-binding domain of arrestin 2 (amino acids 319–418) compete for, and thus act as dominant-negative modifiers of arrestin-fostered, clathrin-coated pit-mediated desensitization (206). Interactions between β-arrestin and AP2 appear important in early phases of endocytosis because arrestin 3 R396A, which cannot interact with AP2, does not support sequestration of agonist-activated β_2-ARs into plasma membrane puncta (207). The role of dynamin in a given endocytosis process is revealed by disruption of the internalization subsequent to concomitant overexpression by a GTP-binding and GTPase-deficient dynamin, K44A or K44E (128, 208).

Because of their high degree of sequence homology and the fact that both arrestin 2 and arrestin 3 desensitize the β_2-AR in vitro, it was once assumed that these two arrestins had indistinguishable functions. However, mouse embryo fibroblasts derived from arrestin 2 (arr2$^{-/-}$), arrestin 3 (arr3$^{-/-}$), or double (arr2$^{-/-}$/arr3$^{-/-}$) knockout mice reveal overlapping, but not identical, roles for these two molecules. It appears that depending on the GPCR, one or the other β-arrestin molecule may be more efficacious in promoting receptor internalization. For example, internalization of the β_2-AR is greatly reduced in arr3$^{-/-}$ MEFs but not in arr2$^{-/-}$ MEFs (209). Angiotensin II $AT_{1A}R$ internalization is not significantly affected in

MEFs lacking either arrestin 2 or 3 but dramatically impaired in MEFs deficient in both arrestin isoforms, suggesting that either arrestin isoform can support $AT_{1A}R$ internalization (209).

In vivo studies in arrestin-deficient mice also reveal the unique functions of arrestin isoforms. Morphine administration to arr3$^{-/-}$ mice leads to enhanced duration of action and analgesic potency (210) and a failure to develop tolerance to repeated administration of morphine. This is in contrast to findings in either wild-type or arr2$^{-/-}$ knockout mice. Interestingly, despite the inability of arr3$^{-/-}$ mice to develop tolerance to morphine, these mice do develop physical dependence on morphine, indicating that these two events are dissociable (211).

Examples of GPCR that manifest arrestin- and dynamin-dependent endocytosis include β_2-adrenergic (212), α_2-adrenergic (213), V2 vasopressin (214), chemokine CXCR4 (215), adenosine 2B (216), μ-opioid (201, 217, 218), proteinase-activated PAR 2 (219), human complement 5a (C5a), anaphylatoxin (CD88) (220), somatostatin (216, 221), and thyroid-releasing (222) receptors. Thus, a large subset of receptors, of which those noted above are only examples, employ a classical GPCR internalization profile, i.e., arrestin- and dynamin-dependent endocytosis in response to agonist.

The nature of interactions between a GPCR and arrestin also determines the rate of receptor recycling. Some GPCRs, such as the β_2-AR, dissociate from arrestin once endocytosed and move into endocytic compartments while arrestin is recycled to the cytosol. These receptors are often rapidly recycled back to the plasma membrane. On the other hand, some GPCRs form a stable complex with arrestin in endocytic compartments. GPCRs more stably associated with arrestin appear to be dephosphorylated less efficiently and are recycled more slowly (214, 223).

ARRESTIN-INDEPENDENT, DYNAMIN-DEPENDENT ENDOCYTOSIS Arrestin-independent but dynamin-dependent internalization is characterized by blockade of GPCR internalization by dominant-negative dynamin, but not by dominant-negative arrestin structures. For example, the $5HT_{2A}$ receptor has a readily measurable constitutive rate of internalization that is accelerated by agonist occupancy. Both forms of internalization are blocked by dominant-negative dynamin, but not by dominant-negative arrestin (224). Agonist provoked internalization of prostacyclin receptors also appears independent of GRK-catalyzed receptor phosphorylation and arrestin binding; in contrast, dominant-negative dynamin partially inhibits receptor internalization (225).

ARRESTIN- AND DYNAMIN-INDEPENDENT ENDOCYTOSIS Arrestin- and dynamin-independent internalization has been reported for the angiotensin II $AT_{1A}R$ (226). However, these findings have not been corroborated by studies in mouse embryo fibroblasts derived from arr2$^{-/-}$ and arr3$^{-/-}$ mice. Angiotensin II AT_{1A} receptor internalization is greatly impaired in these arrestin knockout mice (209). Similarly, RNA interference strategies also are consistent with a role for arrestin in

AT_{1A} receptor internalization (227). Different methodological strategies to assess receptor loss from the surface or appearance intracellularly also can lead to evidence supporting different apparent roles, or not, of dynamin and arrestin (209, 228, 229). Experimental differences in defining a role, or not defining a role, for arrestin or dynamin may reflect the impact of the extent of fractional receptor activation on selecting one versus another GPCR in a pathway for receptor internalization. Because the optimal substrate for GRKs is a comformation such as that induced or stabilized by agonist, arrestin binding to receptors either requires or is significantly enhanced by this GRK-catalyzed phosphorylation; it would not be surprising if there were a direct correlation between relative fractional receptor occupancy and the particular mechanisms selected by a GPCR to contribute to receptor endocytosis in a target cell.

The regions of GPCR sequence involved in interacting with arrestins (e.g., carboxyl tail of G_s-coupled GPCRs, 3i loop of G_i/G_o-coupled GPCRs) are often the same domains involved in interacting with the plethora of other proteins outlined above. The impact of these other GPCR-interacting protein interactions on arrestin-mediated GPCR trafficking and endocytosis represents uncharted terrain. We have observed a reciprocal interplay between spinophilin and arrestin in interacting with the α_{2A}- and α_{2B}-AR subtypes (173); preliminary data suggest that this apparent competition of spinophilin for arrestin may alter the endocytosis profiles of these receptors in native target cells with or without exposure to agonist. Our own data reveal, in fact, that the presence of spinophilin retards internalization of the agonist-occupied α_{2B}-AR subtype, presumably mediated by the binding of arrestin (25). Other GPCR-interacing proteins also may compete with arrestin and other regulatory molecules; these reciprocal interactions remain to be rigorously characterized.

Consequences of GPCR Interactions with Arrestins in Addition to Endocytosis

Arrestin proteins were originally identified based on their role in GPCR desensitization. However, the roles of arrestin have been expanded to include several additional functions in GPCR signaling systems.

One such function is the ability of arrestin in some GPCR systems to serve as an agonist-regulated scaffold for two mitogen-activated protein kinase (MAPK) cascades by linking GPCRs to the components of the extracellular-signal regulated kinase (ERK) cascade (230–232) and the c-jun N-terminal kinase type 3 cascade (JNK3) (233). Data have suggested that only arrestin 3 can scaffold the JNK3 pathway, whereas both arrestin 2 and arrestin 3 can scaffold ERK. For both scaffolds, it appears that the arrestins interact directly with the both MAPKKK and the MAPK, but indirectly with MAPKK. Scaffolding by arrestins appears to play a dual function: (*a*) ensuring precise regulation of the MAPK pathway by colocalizing and facilitating phosphorylation of successive members of the cascade and (*b*) protecting or insulating the MAPK pathway by preventing crosstalk with functionally unrelated members of other kinase signaling molecules (234).

Recent findings indicate that arrestin interaction with the β_2-AR provides a mechanism to directly link the receptor with phosphodiesterase (PDE) 4 (235). Agonist activation leads to membrane recruitment of PDE4, an effect that is lost in mouse embryonic fibroblasts lacking arrestin 2 and 3. Coimmunoprecipitation studies confirm that arrestins can bind PDE4 (and other isoforms). Functionally, PDE recruitment leads to enhanced cAMP degradation and subsequent depression in PKA activity; consistent with this, a catalytically inactive mutant (PDE4D5-D556A) blocks agonist-stimulated PDE4 and leads to elevated PKA activity (235). These interactions appear important in PKA-mediated β_2-AR G_s switching to G_i, measured by assessment of ERK activation (236).

The postulate that receptor-arrestin complexes can trigger additional signaling events such as MAP kinase activation has recently been extended to include apopotosis. The *Drosophila* arrestin protein, Arr2, has been found to play a role in regulating retinal cell apoptosis in the fly. This apoptosis requires rhodopsin phosphorylation, Arr2 phosphorylation by calcium/calmodulin-dependent kinase II (CamKII), and clathrin-mediated endocytosis. Whether this interaction plays a similar role in mammalian retinal cell degeneration is yet to be determined.

The reader is referred to several excellent and comprehensive reviews discussing the impact of signal termination (i.e., G protein signaling) and signal initiation (i.e., arrestin-dependent activation of non-G protein pathways) (194, 237–241).

The Role of NHERF/EBP50 in Agonist-Evoked Receptor Endocytosis and Recycling

Arrestins are not the only protein family that has been implicated in mediating post-GPCR activation events. The interaction of Na^+/H^+ exchange regulatory cofactor (NHERF) with the β_2-adrenergic receptor was identified in a search of PDZ-binding proteins interacting with the C terminus of the receptor (242). These interactions have been extended to the KOR, purinergic P2Y1 and P2Y2, and the parathyroid hormone PTH1R (242–244), albeit via differing PDZ domains within NHERF.

NHERF-GPCR interactions not only have been reported to influence signaling (244–247) but also GPCR recycling. NHERF1 (also known as EBP50) has been demonstrated to facilitate GPCR recycling for the β_2-AR, KOR, and DOR (243, 248, 249). As with the β_2-AR, KOR binding to NHERF is agonist-regulated; this interaction decreases KOR downregulation (with no changes in the affinity of the receptor for ligand nor receptor-G protein coupling) owing to an accelerated recycling of internalized KORs (243). Disruption of β_2-AR-NHERF interactions alters postendocytic sorting, culminating in increased receptor degradation (248). Additionally, fusion of the β_2-AR-NHERF binding region to the DOR transforms this lysosomally targeted GCPR into a recycling receptor (249).

The C-terminal tail of the β_2-AR also represents the sequence region that interacts with NEM-sensitive factor (NSF), a membrane fusion regulatory protein critical for vesicular trafficking in eukaryotic cells (250, 251). However, NSF is

presumed to interact with the β_2-AR by a mechanism distinct from PDZ domain interactions, as NSF lacks a canonical PDZ domain and mutations in the C terminus that perturb NSF binding to the β_2-AR do not perturb PDZ-domain-mediated binding of NHERF to this receptor. The quantitative role of NHERF/EBP versus NSF in GPCR recycling may vary in different cell types or under differing regulatory conditions because a mutant β_2-AR that does not bind NSF (β_2-AR-412A) but binds NHERF is defective in receptor recycling, whereas a mutant β_2-AR that cannot bind NHERF but can bind NSF (β_2-AR-410A) recycles normally (252).

MISLOCALIZATION OF GPCRS LEADS TO DISEASE

As stated above, appropriate receptor localization is required to permit efficient signaling. The functional importance of GPCR localization is emphasized by diseases that ensue from receptor mislocalization and is discussed below.

X-Linked Nephrogenic Diabetes Insipidus

NDI is characterized by the excretion of large quantities of hypotonic urine in the presence of normal circulating levels of the antidiuretic hormone, arginine vasopressin (AVP). In kidney renal epithelia, AVP binds its cognate GPCR, the V2R. AVP binding and coupling via the stimulatory G_s protein elicits cAMP-dependent PKA-mediated fusion of preexisting aquaporin 2–bearing vesicles with the apical membrane. Apical insertion of these water channels affords water reabsorption and the subsequent concentration of urine in renal proximal tubule cells. Patients affected with NDI are unresponsive to AVP and thus experience polyuria and subsequent polydipsia.

The majority of patients ($>$90%) identified with NDI are males with the X-linked form of NDI, and harbor mutations within the V2R open reading frame. Of the $>$150 X-linked mutant human alleles encoding V2R, approximately 70% appear to encode intracellularly retained V2Rs when examined in heterologous cell expression systems (253). Several mutants demonstrate prolonged association with endoplasmic reticulum resident chaperones, such as calnexin (254). Moreover, of the clinically relevant V2R mutants that correctly traffic to the cell surface of renal tubule cells, many display either low-affinity binding for AVP or reduced coupling to activation of adenylyl cyclase. To investigate the impact of V2R mutations in vivo, the clinically identified X-linked NDI nonsense mutation G242X introduced via targeted mutagenesis in mouse embryonic stem cells results in a phenotype indicative of human X-linked NDI (255). V2R hemizygous male pups were unable to concentrate their urine, displayed an enlarged pelvic area, failed to thrive, and died within seven days of birth owing to water loss. In contrast, female mice heterozygous for the G242X V2R ($V2R^{+/G242X}$) displayed normal growth characteristics while exhibiting polyuria and polydipsia. Indeed, saturation binding studies performed on $V2R^{+/G242X}$ kidney preparations demonstrated that the

number of detectable [^{3}H]AVP V2R binding sites was decreased ∼50% without any change in receptor affinity; as expected, V2R-mediated increases in cAMP accumulation were depressed in inner medullary collecting duct preparations from heterozygous $V2R^{+/G242X}$ mice. This mouse represents the first animal model for this disease and may prove useful for assessing therapeutic strategies for the treatment of X-linked NDI, as well as determining the functional consequence of the loss of V2R-dependent events in vivo.

In vitro studies demonstrate that wild-type V2Rs are retained intracellularly when coexpressed with synthetic V2R truncation mutants. Studies performed by Wess and colleagues revealed that coexpression of a wild-type V2R polypeptide encoding half of the 3i loop, TM6, TM7, and the C terminus can functionally rescue a variety of V2Rs mutated in the 3i loop or the last two transmembrane regions, but not a V2R mutant (Y128S) located upstream of the V2(tail) region of complementation (92). The V2(tail) polypeptide coexpressed with individual V2R mutant alleles increased the number of detectable V2R binding sites, as well as increased receptor-stimulated adenylyl cyclase activity. These data support the concept that dimerization with mutant alleles can disrupt wild-type receptor localization, but one might exploit this property to design polypeptides to modify the mutant allele response.

It is also of significant therapeutic interest that several mutant V2Rs can be spatially and functionally rescued in vitro by membrane-permeant antagonist treatment. Incubation of cells heterologously expressing individual V2R mutants with the membrane-permeant V2R antagonist SR121463B facilitates cell surface receptor localization, as assessed via ligand binding, immunofluorescence studies, and receptor-dependent effector coupling (256). Based upon the hypothesis that GPCR ligands stabilize a particular receptor structure, pharmacological chaperones may support a three-dimensional conformation of the V2R that the intracellular folding machinery would otherwise not recognize in the mutant receptor protein.

Selected V2R mutants are also rescued to the cell surface by maintenance at permissive (28°C) temperatures (257), analogous to studies performed with the ΔF508-CFTR (258). Although the mechanisms accounting for pharmacological, chemical, and temperature rescue largely remain unclear, these strategies have been employed to restore proper localization of diverse proteins in addition to mutant V2Rs, including the mutant Z-variant of α1-antitrypsin (259), mutant HERG (260), and ΔF508-CFTR molecules (258). Collectively, these observations strengthen the concept that misfolded structures and protein mislocalization can lead to the disease state.

Retinitis Pigmentosa

Phototransduction couples photon energy to a visual image with rhodopsin as the initial receptor for light. Infrared-laser atomic-force microscopy reveals that, in its native state, rhodopsin exists in ordered, paracrystalline dimeric arrays (261, 262). The rhodopsin signal transduction cascade is initiated by multiple electronic

isomerizations of 11-*cis*-retinal covalently attached via a Schiff base to its seven transmembrane–spanning GPCR, opsin (263). Subsequent rhodopsin-dependent coupling via the heterotrimeric G protein transducin (G_t) leads to activation of a cGMP-activated phosphodiesterase enzyme. Depression of cGMP levels results in closure of cGMP-gated channels, retinal rod hyperpolarization, signal transmission, and ultimate integration of visual information.

Retinitis pigmentosa (RP) is a hereditary, genetically heterogeneous retinal disease characterized by progressive photoreceptor degeneration and eventual retinal dysfunction. RP is a complex, multifactorial disease with mutations in numerous gene products (∼36 known or predicted RP genes) encoding nearly all of the components of the visual signaling pathway, including rhodopsin, transducin, the α and β subunits of cGMP phosphodiesterase, the α-subunit rod cGMP-gated channel, photoreceptor structural genes, and transcription factors, among other downstream signaling molecules [reviewed in (264)].

One form of RP is comprised of individuals harboring mutations within the gene encoding rhodopsin. Mutations in rhodopsin constitute a substantial fraction of autosomal dominant RP (ADRP); currently, the vast majority of these mutant alleles (categorized as Class II or Class III) harbor mutations within the transmembrane helices and/or extracellular domains resulting in a mutated receptor molecule that is retained intracellularly with no or negligible 11-*cis*-retinal binding (265). In contrast, Class I mutants carry mutations within the first transmembrane domain and/or extreme carboxyl tail and interfere with normal receptor targeting to the rod outer segment despite retaining 11-*cis*-retinal binding (265–267).

The majority of the ∼150 opsin gene mutations represent rare occurrences, except for the P23H Class III mutation; this substitution mutant constitutes ∼10% of all ADRP cases and represents a founder mutation within the North American population (268). Intracellularly retained P23H opsin molecules form aggregates that include complexes with endoplasmic reticulum chaperones (269); P23H opsin can serve as a negative regulator of wild-type function by recruiting the normal opsin to aggresomes, suggesting a mechanism for disease dominance (93).

Pharmacological rescue of the P23H opsin allele has been demonstrated with the 11-*cis*-retinal analog, 11-*cis*-7-ring-retinal. In heterologous cells, 11-*cis*-7-ring retinal restored proper P23H folding, fostered acquisition of a mature glycosylated receptor state, and rescued expression to the cell surface (270). In contrast to the covalent attachment of 11-*cis*-retinal to the wild-type opsin structure, however, the retinoid analog is liberated over time from the P23H mutant opsin. Nonetheless, these findings are consistent with the concept that pharmacological chaperoning may foster or stabilize a three-dimensional structure that maintains a wild-type-like conformation conducive for proper folding, allowing the mutant protein to escape the degradation pathway and attain cell surface expression. Furthermore, these data emphasize that disease states resulting from GPCR misfolding and aberrant trafficking itineraries may be amenable to therapeutic intervention.

Findings in streptozotocin-induced diabetic rats are of potential therapeutic interest for mutations in rhodopsin, in particular, and for misfolded protein diseases

in general. Streptozotocin-treated rats represent an animal model displaying morphological and functional alterations in the retinal vasculature similar to human diabetic retinopathy, a retinal disorder akin to RP. Treatment of these animals with the drug bimoclomol or its analogs improves indices of retinal function, an effect attributed to the cytoprotective properties of bimoclomol. Of notable interest is that bimoclomol stimulates heat shock protein-70 (HSP70) expression; because induction of HSP70 previously has been shown to rescue ΔF508-CFTR to the cell surface (271), these findings collectively imply that bimoclomol or its analogs may represent potential therapeutic compound candidates for the treatment of certain forms of ADRP.

Hypogonadotropic Hypogonadism

GnRH secreted from the hypothalamus induces hormonal gonadotropin [follicle stimulating hormone (FSH), luteinizing hormone (LH)] synthesis and release from pituitary gland gonadotropes. In response to FSH and LH, the sexual organs (ovaries and testes) release additional hormones ultimately responsible for sexual development. Any disruption in this pathway results in sexual dysfunction and impairs sexual maturation. Hypogonadotropic hypogonadism (HH) is characterized by absent or decreased gonadal function and manifests as delayed sexual development in affected individuals. One form of HH ensues from mutations within the GnRH receptor (GnRHR) gene. Receptor mutations widely dispersed within the GnRHR coding region have been identified from affected individuals demonstrating a wide range of clinical phenotypes (272); heterologous expression of these GnRHR alleles reveals that the majority encode misfolded receptors with an increased propensity for targeted degradation or intracellular retention. Like many GPCRs, the GnRHR homodimerizes, based on fluorescence resonance energy transfer (FRET) studies (273). The isolation and identification of a GnRHR truncated splice variant lacking TM6 and TM7 from human pituitary specifically inhibiting wild-type GnRHR-mediated signaling in vitro underscores the possibility for a mechanism of disease dominance (94), i.e., mutations (point mutations or truncations) functioning as negative regulators of wild-type function.

In contrast to cloned nonmammalian GnRHR members (and indeed all other GPCRs), the mammalian (rat) wild-type GnRHR lacks a predicted intracellular carboxyl tail (274). Synthetic mutations of the extreme carboxyl region of the mammalian GnRHR reveal residues important in ligand binding and effector coupling in the absence of alterations in receptor density. Conn and colleagues exploited the disparity between mammalian and nonmammalian GnRHRs by generating a chimeric receptor containing the noncarboxyl tail–containing rat GnRHR fused to the catfish GnRHR carboxyl tail. Addition of the carboxyl tail to the wild-type rat GnRHR increased the number of detectable rat GnRHR binding sites without any change in receptor binding affinity. Moreover, the carboxyl tail increased receptor-mediated inositol phosphate production, via G_q, whereas receptor-mediated increases in cyclic AMP, viaG_s, remained unchanged (274).

Since most clinically identified GnRHR mutant alleles code for receptors with attenuated expression, it would be of interest to determine if sequences with the catfish GnRHR carboxyl tail could be exploited to enhance mutant GnRHR surface expression, particularly for mutant GnRHR alleles that retain ligand binding and coupling to G proteins. However, because the native mammalian GnRHR completely lacks a carboxyl tail, complementation would require additional upstream sequences by analogy with functional rescue of mutant alleles of the V2R by receptor fragment coexpression (see above).

In addition to functional complementation, studies have demonstrated that mutant receptors (synthetic and clinically identified GnRHR alleles) are amenable to pharmacological rescue (275–277). Incubation of cells heterologously expressing individual GnRHR mutants with the membrane-permeant GnRHR antagonist IN3 [(2*S*)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-1*H*-indol-3-yl]-*N*-(2-pyridin-4-ylethyl)propan-1-amine] facilitates mutant receptor expression, as assessed via [^{125}I]-buserelin ligand binding and mutant receptor-dependent effector coupling (275, 276). Further studies have revealed additional membrane-permeant, chemically distinct, GnRHR peptidomimetic agents that rescue naturally occurring and synthetically designed GnRHR mutants (277). Collectively, the data strongly support the importance of receptor misfolding as a contributor to the HH disease and emphasize the generalizable principle of small-molecule pharmacological chaperones.

SUMMARY

As stated in the beginning, for GPCRs to appropriately mediate responses to hormones, neurotransmitters, local mediators, and other endogenous agents, these receptors must achieve their proper localization as nascent cell surface molecules; remain at the appropriate location to respond to regulatory agents, such as endogenous or pharmaceutical agonists; and then engage in regulated turnover. It is likely that a number of GPCR interactions with other proteins make possible and modulate these processes. In fact, common motifs or domains of GPCRs may be critical for interacting with ER and Golgi chaperones in achieving cell surface localization, for tethering at the cell surface, and for eliciting agonist responses and ultimately agonist-modulated cell surface turnover. The connections between all of these processes have not yet been made for all GPCRs, but many of the partial reactions are becoming understood. The plethora of GPCR interacting proteins, some known and others likely to be revealed, argues for transition from studies of cells heterologously expressing an overabundance of GPCRs and/or interacting proteins relative to native cell receptor expression to cells engineered to mimic more natural receptor and interacting protein density. In this way, the stoichiometric interplay of a variety of proteins that interact with GPCRs to modulate or participate in GPCR trafficking and signaling can be discovered. We hope this review serves as a useful progress report in understanding

the important role GPCR trafficking events play as a background for GPCR signaling.

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