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# Adenosine Receptors Find a New Partner and Move Out

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Running title: Adenosine receptors move out with Usp4

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Abbreviations: GPCR, G protein-coupled receptor; $A_{2A}R$, adenosine $A_{2A}$ receptor; ER, endoplasmic reticulum

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## ABSTRACT

Recent studies of G protein-coupled receptors have highlighted two "new" and interactive elements involved in their function and regulation, their ability to localize to different cell surface and intracellular compartments and their ability to interact with partners other than their classic heterotrimeric G proteins. The effects mediated by these receptors can be markedly different depending on the compartment in which they reside and the partners with which they interact in each compartment. The studies in this issue of *Molecular Pharmacology* by Milojevic *et al.* (page #) merge these two themes by identifying the ubiquitin-specific protease Usp4 as a partner for the carboxyl-terminal tail of adenosine $A_{2A}$ receptors and showing that this interaction allows processing and delivery to the cell surface of newly synthesized $A_{2A}$ receptors, which are otherwise predominantly intracellular. Their studies suggest that the intracellular $A_{2A}$ receptors are ubiquitinated, presumably due to misfolding and intervention of the "endoplasmic reticulum quality control" mechanism leading to degradation of the receptors by proteasomes. Increasing Usp4 expression stimulates receptor de-ubiquitination and increases cell surface expression of functional receptors. Evidence is presented for tight specificity of this interaction, with another Usp family member failing to rescue $A_{2A}$ receptors and Usp4 failing to rescue another intracellular receptor. The background and significance of this study are highlighted here, including puzzles that remain to be solved and the potential for pharmacological targeting of such interactions to manipulate the expression, location, and function of G protein-coupled receptors for therapeutic benefit.

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The large and diverse family of G protein-coupled receptors (GPCRs) includes the target molecules for well over half of the medicines in clinical use today; as a result, GPCRs continue to be the targets of extensive investigation by those in the field of molecular pharmacology as well (Pierce, et al., 2002). Two important and overlapping new themes have emerged from GPCR studies over the past few years: 1) their ability to interact with a diverse set of "partners" other than their well-established ligands and G proteins; and 2) their ability to exhibit unique and tightly regulated functions depending on their localization in specialized cell surface microdomains and in multiple intracellular compartments. The studies of the $A_{2A}$ adenosine receptor ($A_{2A}R$) presented in this issue by Milojevic *et al.* (Milojevic, et al., 2006) extend these emerging themes by identifying the ubiquitin-specific protease Usp4 as a new binding partner for the carboxyl-terminal tail of the $A_{2A}R$ and showing the importance of this partnership in allowing the $A_{2A}R$ to move out from its intracellular site of synthesis and assembly to its proper cell surface location for ligand binding and signal generation.

*The right partner at the right place.*

The beta-2 adrenergic receptor and other monoamine receptors have served as the prototypes for understanding the structure, function, and regulation of GPCRs (Pierce, et al., 2002). Studies of their function uncovered the molecular basis for their specific binding of appropriate extracellular ligands and their activation of selective G proteins to generate the intracellular signals that mediate their physiological and clinical effects. Cloning studies for these receptors provided our initial two-dimensional view of the seven-transmembrane domain structure of GPCRs and specific roles for their transmembrane domains and connecting loops both inside and outside the cell, for their extracellular amino termini, and for their intracellular carboxyl-terminal tails. Three-dimensional structures are finally becoming available to both confirm and clarify this picture (Palczewski, et al., 2000).

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The importance of studies of agonist-induced desensitization in expanding our view of GPCRs in general, and in setting the stage for the studies by Milojevic *et al.* (2006) in particular, deserves to be highlighted. Investigating the basis for desensitization revealed the remarkable ability of cells to fine-tune their use of GPCRs by changing levels of receptor expression, by covalent modifications to alter receptor binding and/or signaling properties, and by moving receptors from cell surface to intracellular compartments. Exploring the basis for functional "uncoupling" of receptors from G proteins without changes in receptor number or location revealed the ability of GPCRs to interact with and be modified by multiple protein kinases and the adaptor protein β-arrestin (Lefkowitz, 1998). These studies have been followed with the identification of a now bewildering array of additional protein partners for GPCRs, including intracellular adaptor molecules and effectors for G protein-independent signals (Ali, et al., 2000; Lefkowitz and Shenoy, 2005), scaffolding proteins to both assemble and localize tightly regulated signaling complexes (Hall and Lefkowitz, 2002; Malbon, et al., 2004), and dimerization of GPCRs with each other (Rios, et al., 2001; Milligan, 2004) and with various one- and two-transmembrane domain partners (Bermak and Zhou, 2001). It was also from early studies of desensitization that we first learned that GPCRs were not static in terms of their localization in the bulk plasma membrane but could instead be moved to clathrin-coated pits for endocytosis and further processing (Perkins, et al., 1991; Ferguson, 2001). Their apparent movement in and out of caveolae/rafts and other plasma membrane microdomains provides yet another complexity in terms of both localization and function (Ostrom and Insel, 2004; Neve, 2005). The trafficking of GPCRs through a complex network of intracellular vesicles to be either recycled to the surface or degraded in lysosomes or by proteasomes is an ongoing focus of research, including studies of the GPCR partner proteins that may chaperone their movement among these compartments (Rosenfeld, et al., 2002; von Zastrow, 2003).

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*Usp4 helps $A_{2A}$Rs move out.*

The studies by Milojevic *et al.* (2006) highlighted here begin with the unexpected finding of a predominantly intracellular localization of endogenously expressed $A_{2A}$Rs in PC12 cells reported previously (Arslan, et al., 2002), and they lead to identification of the ubiquitin-specific protease Usp4 as a protein partner that is critical for moving newly synthesized receptors out to the cell surface. The findings are important for understanding normal cellular processing of GPCRs, because much less is known about how these molecules are properly folded and delivered to the cell surface for the first time following their synthesis (Duvernay, et al., 2005), in comparison to all that is known about their subsequent rounds of endocytosis, recycling, and down-regulation. Several pathologies are known to result from misfolding of newly synthesized transmembrane proteins and the strong intervention of an endoplasmic reticulum (ER) "quality control" mechanism to ensure that misfolded proteins are not delivered to the cell surface, even though they may be functional (Kostova and Wolf, 2003; Ye, 2005). The ΔF508 mutation of the CFTR transporter in cystic fibrosis is the prototypical clinical example, and the defective $V_2$ vasopressin receptor involved in nephrogenic diabetes insipidus and mutations of the gonadotropin-releasing hormone receptor involved in hypogonadism are among the best characterized models for GPCR folding and delivery (Ulloa-Aguirre, et al., 2004; Bernier, et al., 2004). There is evidence for the effectiveness of pharmacological manipulations to circumvent this ER quality control mechanism, by either chaperoning the more correct folding of these proteins or by promoting delivery of the misfolded proteins to the surface where their reduced but partial functionality may be better than the total lack of protein delivery and function enforced by ER quality control. For a variety of defective GPCRs, improper folding can be rescued by "chemical chaperones" such as glycerol and DMSO or by more selective "pharmacological chaperones", including the normal ligand for the receptor (Ulloa-Aguirre, et

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al., 2004; Bernier, et al., 2004). In many cases, defective and misfolded proteins become ubiquitinated and are thereby targeted to the proteasome for degradation (Kostova and Wolf, 2003; Ye, 2005). The new studies in this issue provide evidence that the intracellular $A_{2A}$Rs are in fact ubiquitinated, presumably as part ER quality control, and that increased expression of the ubiquitin-specific protease Usp4 increases the amount of non-ubiquinated $A_{2A}$R and allows or promotes delivery of these receptors to the cell surface. After they move out to the cell surface with the help of Usp4, these receptors are capable of apparently normal binding and G protein-mediated signaling. Pharmacologic manipulation of the ubiquitinating and de-ubiquitinating enzymes involved in ER quality control can thus be added to the list of potential therapeutic options for rescuing partially defective and misfolded GPCRs and other proteins that may contribute to various pathologies.

The authors first show that proteasome inhibition allows a greater fraction of the receptor molecules to become or remain functional, and they identify a specific segment of the intracellular carboxyl terminus of the $A_{2A}$R that is required for this effect. They next use yeast two-hybrid screening of a human brain RNA library to identify partner proteins for the receptor tail that might be involved in targeting the $A_{2A}$R to the proteasome and preventing its movement to the surface. The ubiquitin-specific protease Usp4 was one of several molecules identified in their screen, and it was chosen for more detailed study because of the known role of ubiquitination in proteasome-mediated protein degradation and ER quality control and the ability of proteasome inhibitors to increase $A_{2A}$R expression. The direct interaction of Usp4 with the $A_{2A}$R is clearly documented and shown to be specific for the same portion of the $A_{2A}$R tail that is required for increasesd expression by proteasome inhibition. This interaction is important for surface trafficking of the $A_{2A}$R also, because increasing Usp4 expression increases cell surface delivery and decreases the intracellular pool of the full-length $A_{2A}$R but not of the tail-truncated

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receptor construct. Involvement of the de-ubiquitinating activity of Usp4 in this effect is supported by the fact that the Usp4-enhanced increase in surface receptors is accompanied by removal of tagged ubiquitin from the receptor and a decrease in apparent size of the receptor from 48-50 kDa to 40-42 kDa (the expected size difference for mono-ubiquitination). Proteasome inhibition still led to an increase in $A_{2A}R$ expression levels and cell surface delivery in cells over-expressing Usp4, suggesting that even in the presence of high levels of Usp4 there continues to be significant delivery of these receptors to the proteasome for degradation.

Milojevic *et al.* (2006) address several potential caveats, which helps to clarify and strengthen their basic conclusions. They show that the intracellular localization of $A_{2A}Rs$ is not limited to the PC12 cells in which their initial studies were conducted; endogenous $A_{2A}Rs$ in hippocampal neurons are also predominantly intracellular, and transfection with Usp4 moves more $A_{2A}Rs$ to the surface in these cells as well. The effects of Usp4 are not artefacts of its artificial overexpression, because utilizing siRNA to decrease the expression of endogenous Usp4 mRNA in PC12 cells increased the intracellular accumulation of $A_{2A}Rs$. These data increase the likelihood that a relevant cellular mechanism for controlling the fate of newly synthesized $A_{2A}Rs$ and perhaps other GPCRs has been discovered. Because ubiquitination is known to play a role in endocytosis of cell surface GPCRs and their subsequent degradation, particularly in yeast but also in mammalian cells (Hicke, 1999; Shenoy, et al., 2001; Wojcikiewicz, 2004), the authors addressed the question of whether the intracellular $A_{2A}Rs$ were newly synthesized and had never been delivered to the cell surface, or were instead trapped intracellularly following endocytosis from the surface. The intracellular ubiquitinated receptors were endoglycosidase H-sensitive whereas the surface receptors in Usp4-transfected cells became endoglycosidase H-resistant, indicating that the intracellular $A_{2A}Rs$ have not completed

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their processing and presumably have never left their site of synthesis because of improper folding and subsequent ubiquitination.

From a pharmacological perspective, the specificity of the interaction between Usp4 and the $A_{2A}R$ demonstrated in this study is particularly intriguing. The authors show that the closely related Usp14 protein does not rescue $A_{2A}R$ cell surface delivery; similarly Usp4 does not rescue cell surface delivery of the mGluR5 metabotropic glutamate receptor, which is also found to be predominantly intracellular. How many and which of the other Usp family members are involved in ER quality control and/or moving out of how many and which other GPCRs? Recent work indicates that there are more than 50 members of this Usp protein family (Quesada, et al., 2004), enough for fairly selective interactions with small sets of GPCRs. Why the cell would need so many different Usp proteins for its many different GPCRs is not clear. However, separate control of GPCR synthesis and cognate Usp protein synthesis and/or activity would allow for potentially rapid and tightly regulated control of which GPCRs are expressed at the cell surface under changing conditions. If this is the case, what are the mechanisms that in turn regulate the expression and action of the GPCR-regulating Usp proteins? The potential that specific Usp proteins can be pharmacologically manipulated to modulate expression of specific GPCRs for therapeutic benefit is an exciting new direction that merits further investigation.

*Puzzles and possibilities*

There are several puzzling aspects of the studies that should also be mentioned. The receptor being studied appears to be the normal wild-type $A_{2A}R$ and not a mutated or defective receptor. Furthermore, this receptor both binds radioligand and generates G protein signals normally once its surface delivery is accomplished. Why do multiple cell types nonetheless fail to allow delivery of this receptor to the surface? Why is this receptor misfolded if its amino acid sequence is normal? Or why is it ubiquitinated and retained intracellularly if it is not misfolded?

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Are there advantages for the cell in synthesizing these receptors but maintaining them in an intracellular pool? Why are endogenously expressed levels of Usp4 not sufficient to allow these functional receptors to be delivered? One possibility is that these receptors serve an important function from their intracellular location, perhaps responding to cytosolic adenosine. Equally attractive is the possibility that these receptors are being held in reserve for rapid delivery to the surface under specific conditions when their activity is needed, a process that could be regulated by control of Usp4 expression or activity. Is it possible or likely that all GPCRs have Usp family proteins as partners following their normal synthesis, to chaperone their folding and surface delivery? Another set of questions not yet answered is precisely where and when Usp4 interacts with the $A_{2A}R$. Does Usp4 accompany the receptor on its path to the cell surface, or only act as a gatekeeper to control whether or not the receptor leaves its site of assembly? Might Usp4 or other Usp proteins also be involved in the endocytosis of GPCRs and their subsequent intracellular trafficking back to the surface or delivery to lysosomes or proteasomes for down-regulation?

As with most new discoveries, the studies by Milojevic *et al.* (2006) raise as many important questions as they answer. Fortunately, tools to address these questions are available, so further insights into these and other questions regarding GPCR synthesis, insertion, folding, and delivery should be on the horizon. The potential for new pharmacological approaches to manipulate which receptors are expressed at the surface of specific cells, together with more classical pharmacologic approaches to then manipulate the activity of those receptors, could make for highly specific new therapies.

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## FIGURE LEGEND

Figure 1. Model of Usp4 rescue of $A_{2A}R$ cell surface delivery and function. Left side: The seven-transmembrane $A_{2A}R$ molecule is located intracellularly in the absence of adequate levels of Usp4 (low endogenous levels or following depletion by RNA interference), presumably retained in the ER (brown) due to the stringent "ER quality control" system. The $A_{2A}R$ is depicted as misfolded and tagged with ubiquitin (Ub), which both prevents its further processing and trafficking to the cell surface and contributes to its delivery to the proteasome (blue) for degradation instead. Right side: With Usp4 expression increased to adequate levels (higher endogenous levels or following transfection), Usp4 binds to the intracellular carboxyl-terminal tail of the $A_{2A}R$ to promote its de-ubiquitination. This allows more receptors to be processed through the Golgi and move to the cell surface and correspondingly decreases the intracellular receptor pool. Though it is not clear whether the cell surface $A_{2A}R$ delivered in this manner is entirely properly folded, it is functional for both binding and signaling, thus rescuing adenosine responsiveness for the cell.

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