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Regulation of G Protein and Mitogen-Activated Protein Kinase Signaling by Ubiquitination: Insights From Model Organisms

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Ubiquitin and Ubiquitin-Like Proteins in Protein Regulation

Seven-Transmembrane Receptors and Ubiquitination

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Regulation of G Protein and Mitogen-Activated Protein Kinase Signaling by Ubiquitination

Insights From Model Organisms

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***Abstract*—**Guanine nucleotide binding proteins (G proteins) and mitogen-activated protein kinases are highly conserved signaling molecules engaged in a wide variety of cellular processes. The strength and duration of signaling mediated by G proteins and mitogen-activated protein kinases are well known to be regulated via phosphorylation of pathway components. Over the past few years, it has become evident that many of the same signaling proteins also undergo ubiquitination, a posttranslational modification that typically leads to protein degradation. Consequently the strength and duration of signaling can also be modulated by regulating the abundance of signaling proteins. This article describes G protein– and mitogen-activated protein kinase–mediated signaling pathways that are known to be regulated by ubiquitination. The focus is on studies performed in the budding yeast *Saccharomyces cerevisiae*, as many principles governing this new regulatory mechanism were initially discovered in this model organism. Similar mechanisms uncovered in other model systems are also briefly discussed to illustrate the importance and universality of signaling regulation by ubiquitination. **(*Circ Res*. 2006;99:1305-1314.)**

Key Words: GPCR ■ G proteins ■ MAP kinases ■ ubiquitination ■ yeast

Many signals are transmitted from the cell surface to intracellular effectors through the actions of guanine nucleotide binding proteins (G proteins). On agonist binding to its receptor, the G protein α subunit releases GDP, binds GTP, and dissociates from the G-protein $\beta\gamma$ subunits. Depending on the system, either Gα or G$\beta\gamma$ can then activate downstream effectors, such as ion channels, adenylyl cyclase, phospholipases, or protein kinases such as components of the mitogen-activated protein kinase (MAPK) cascade.[1] The basic assembly of a MAPK module consists of 3 distinct kinases, which are activated sequentially through a series of phosphorylation events.[2,3] MAPKs phosphorylate diverse substrates including nuclear transcription factors, cytoskeletal proteins, as well as downstream kinases, to regulate gene expression, cell division, cell morphology, and cell survival.[4]

Signaling pathways mediated by G proteins and MAPKs are involved in the cellular responses to an enormous array of stimuli, including hormones, cytokines, antigens, neurotransmitters, stress, as well as to changes in extracellular matrix and cell-cell contacts.[2,4] A large number of cellular processes, such as proliferation, differentiation, and apoptosis, depend on the activation state of MAPKs. Perturbation of MAPK signaling can lead to a multitude of human diseases and debilitating conditions such as pain, inflammation, cancer, cardiac hypertrophy, and cardiac arrest.[5–7] Given the importance of MAPK signaling to human pathogenesis, enzymes in

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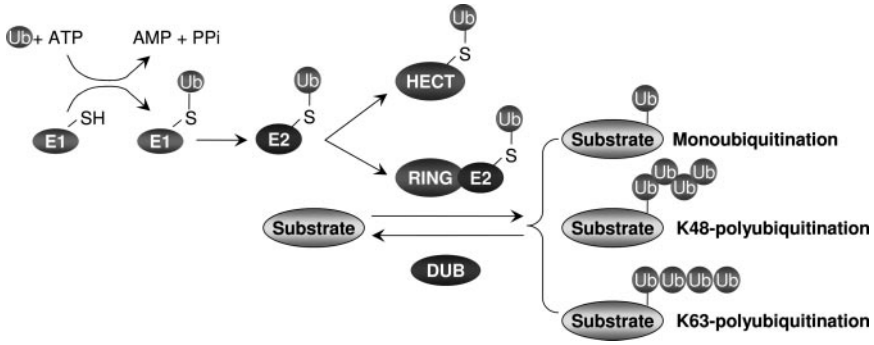

Figure 1. Protein ubiquitination pathway. Ubiquitin is conjugated to its substrate proteins in a reaction that require 3 enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). E1 activates free ubiquitin via the formation of a thiolester bond between its active site Cys and the C-terminal carboxyl group of ubiquitin; E2 and E3 transfer the activated ubiquitin to a substrate. Of the 2 main families of E3s, the HECT E3s form a thiolester bond with ubiquitin before transferring it to a substrate, whereas the RING E3s function as a bridge between an activated E2 and its substrate. Ubiquitin can conjugate to its various substrate proteins, either singly or in chains. The 2 internal lysine residues in ubiquitin, ie, K48, and K63, are commonly used to link the ubiquitin molecules to each other. Conjugated ubiquitin or ubiquitin chain can be removed from the substrates by the action of DUBs. Ub indicates ubiquitin.

MAPK pathways are being actively pursued as potential therapeutic targets.[8–10] For instance, a highly selective inhibitor of p38 MAPK, has been successfully developed and is in clinical trials for the treatment of acute coronary syndromes.[10] A more thorough elucidation of how MAPK signaling is regulated would be useful in revealing novel disease mechanisms and could dramatically aid in our ability to better define targets for therapeutic intervention.

An important mechanism for the regulation of G protein– and MAPK-mediated pathways is posttranslational modification. In this regard, much effort has focused on feedback phosphorylation of receptors and effector kinases.[4,11] Growing evidence indicates that signaling components also undergo ubiquitination, a posttranslational modification in which the modifier is a small protein called ubiquitin (Figure 1).[12–14] Elegant work performed in budding yeast, as well as in mammalian cells, demonstrated that many cell-surface receptors are modified by ubiquitin, and in most cases ubiquitination is essential for sorting receptors to the lysosomal pathway for degradation.[15–20] Evidence accumulated over the past few years indicates that a variety of downstream components are also subject to regulation by ubiquitination. In yeast, the postreceptor components demonstrated to be ubiquitinated include a G-protein α-subunit Gpa1,[21] a regulator of G-protein signaling (RGS) Sst2,[22] and a MAPK kinase (MEK) Ste7[23] (Figure 2). Similarly in mammals, several G-protein subunits,[24–28] 4 RGS proteins,[29–32] 1 MEK kinase (MEKK),[33] and several MAPK isoforms[34–36] have been reported to undergo ubiquitination. Therefore, regulation of signaling components by ubiquitination is a highly prevalent phenomenon, and a thorough understanding of the mechanism and physiological consequences of these modification events would provide a better understanding of cell regulation.

Mechanism of Ubiquitination

Ubiquitin is a highly conserved 76-aa polypeptide that carries out all of its functions by becoming covalently conjugated to specific target proteins. Conjugation of ubiquitin to a substrate requires a cascade of reactions catalyzed by at least 3 distinct enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligase (E3)[13,37] (Figure 1). First, a ubiquitin molecule is activated by E1 via the formation of a high-energy thioester linkage between its C-terminal glycine residue and a catalytic cysteine residue of the E1; once activated, the thiol-linked ubiquitin is transiently transferred to a catalytic cysteine residue on the E2; finally, an E3 ubiquitin ligase facilitates the transfer of ubiquitin from the E2 to the substrate. Based on their distinct mechanisms and structural features, E3 ubiquitin ligases can be divided into 2 major classes: (1) proteins with a HECT (Homologous to E6-AP Carboxyl

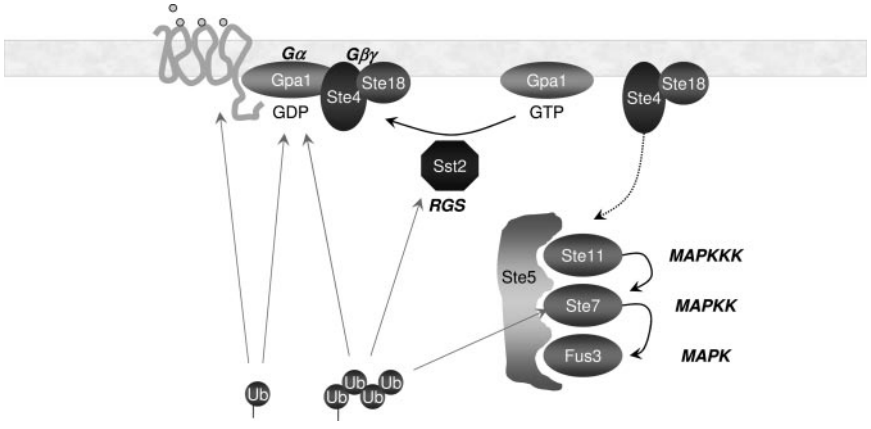

Figure 2. Components of the yeast pheromone response pathway that undergo ubiquitination. Three proteins in the pathway, ie, a Gα Gpa1, a RGS protein Sst2, and a MAPKK Ste7, have been shown to be regulated by ubiquitination. Gpa1 undergoes both mono- and polyubiquitination, whereas Sst2 and Ste7 are modified by polyubiquitination.

Terminus) catalytic domain; and (2) proteins with a RING (Really Interesting New Gene) domain[38,39] (Figure 1). The HECT E3s directly participate in catalysis by providing a catalytic cysteine residue, whereas the RING domain E3s facilitate the ubiquitination process by recruiting ubiquitin-charged E2 to the substrate.[37,40] RING E3s can be further subdivided into 2 classes: those containing only a single polypeptide that possesses both a RING domain and substrate recognition motifs and those containing multiple polypeptides in which the RING domain and substrate recognition motifs reside in distinct subunits.[13,37]

In addition, proteins with domains, such as the U box and the PHD (Plant HomeoDomain), that are similar but not identical to RING have also been demonstrated to have E3 ligase activity.[34,41] The U box is predicted to have a 3D structure similar to that of a RING domain but does not have the conserved cysteine residues,[41] whereas the PHD domain has 8 similarly but not identically spaced cysteines and histidines as in the RING domains.[34]

Akin to phosphorylation, the ubiquitination process is highly selective and specific. The specificity of ubiquitination is afforded by the large number of E3 ubiquitin ligases.[13,37] In most eukaryotes, there is only 1 E1, a limited number of E2s (11 in yeast, and ≈60 in human), and a large number of E3s (54 in yeast, and nearly 400 in human cells by a recent estimate).[37,42,43] Each E3 recognizes a distinct set of substrates and acts in concert with appropriate E2s to catalyze substrate ubiquitination.[37] In many cases, recognition by E3 ubiquitin ligases appears to require phosphorylation of the substrate protein.[39,44] Given the overwhelming prevalence of phosphorylation in signaling pathways, and the close relationship between phosphorylation and ubiquitination, it is no surprise that the ubiquitination system is now emerging as a major player for signaling regulation.

The predominant role of ubiquitination is to target substrates for rapid degradation within a large protease complex, the 26S proteasome.[13] In order for this to occur, usually the substrate is modified with a polyubiquitin chain consisting of a minimum of 4 ubiquitin molecules linked through lysine 48 (K48) on ubiquitin itself, which can then be efficiently recognized by specific ubiquitin receptors on the proteasome.[45,46] New discoveries indicate that ubiquitination does not always lead to degradation of the substrate and it can in some cases play a much broader role in regulating protein function by proteasome-independent processes.[14,47] Perhaps the best-studied example is ubiquitination acting as a signal for membrane protein trafficking.[48,49] In yeast and in mammalian cells, monoubiquitination is a necessary and sufficient signal for internalization of many cell-surface proteins and entry into the endocytic pathway.[16,50,51] More recent studies have also revealed novel roles for ubiquitination in DNA repair and in the activation of IκB kinase.[52,53] In the latter case, ubiquitination of TRAF6, an upstream component in the IκB pathway, leads to recruitment and activation of TAK1 kinase complex, which in turn activates IκB kinase.[54,55] The distinct role of ubiquitination conferred on TRAF6 appears to stem from the unique linkage of the ubiquitin chain it possesses. Instead of a polyubiquitin chain linked through K48 on ubiquitin, TRAF6 is conjugated with a polyubiquitin chain linked through K63.[55] Notably, ubiquitin has 5 additional lysine residues, ie, K6, K11, K27, K29, and K33. Proteomic studies performed in yeast indicate that those residues can also be used to form polyubiquitin chains.[56] Thus the existence of multiple distinct sites of ubiquitin/ubiquitin conjugation represents a mechanism to increase the functional diversity of polyubiquitination.

Deubiquitination of Substrates

Similar to phosphorylation, ubiquitination is reversible. Removal of ubiquitin or ubiquitin chains from substrates is catalyzed by deubiquitinating enzymes (DUBs), a family of proteases that belong to the cysteine protease superfamily[57] (Figure 1). In yeast, there are 17 DUB family members, with sizes ranging from 30 to over 200 kDa; in humans, there are ≈95 DUBs.[58] Outside of the conserved catalytic core regions, there is tremendous diversity among the different DUBs.[58] The large number and diversity of DUBs suggests that there are highly specific functions for each family member, and they may function in specific signaling or developmental pathway(s). Indeed, work from both yeast and *Dictyostelium discoideum* indicates that MAPK signaling is regulated by specific DUB isoforms, and the regulation appears to occur at the level of the MAPK cascade.[23,59]

Yeast uses a MAPK cascade to mediate its responses to the mating pheromone.[60] Interestingly, disruption of just 1 DUB, Ubp3, enhances the yeast pheromone response and promotes pheromone-dependent ubiquitination of a MEK Ste7 in the pathway[23]; similarly, in *D discoideum,* a specific DUB, UbpB, reverses the ubiquitination of MEKKα and stabilizes the protein.[59] Given the prevalence of ubiquitination of signaling components and regulators, a systematical characterization of the functions of those DUBs by either genetic and/or biochemical approaches would likely reveal new mechanisms for cell regulation.

Ubiquitination and G Protein–Coupled Receptor Trafficking: It All Started With Pheromone Receptors

In terms of regulation of G protein–coupled receptor (GPCR)-initiated signaling, the most extensively studied ubiquitin substrates are GPCR themselves. For this particular topic, numerous excellent review articles are available.[48,49,51,61,62] Thus here we only briefly discuss the classic studies in this area, which were performed initially in yeast, to illustrate how lessons learned from simple model systems can reveal fundamentally important mechanisms of signal regulation in humans.

Once activated, most cell-surface receptors, including nearly all characterized GPCRs, are downregulated by removal from plasma membranes via endocytosis.[63] The first evidence indicating that ubiquitination facilitates this process came from studies of yeast pheromone receptors.[16,17] Yeast has 2 haploid cell types, known as MATα and MATa, each of which secrete small peptide pheromones, called α factor and a factor, respectively. The α-factor pheromone binds to a specific receptor (Ste2) on MATa cells, whereas a factor binds to a distinct receptor (Ste3) on MATα cells. On ligand binding, the pheromone receptors activate a signaling path-

way mediated by a heterotrimeric G protein; the receptors subsequently undergo rapid internalization and degradation in the vacuole (yeast counterpart of the mammalian lysosome) for the purpose of downregulating the pheromone signal.[60] Interestingly, both pheromone receptors Ste2 and Ste3 are modified by a single ubiquitin (monoubiquitination) on a lysine reside within their intracellular C-terminal tail.[16,17,50] Ubiquitination of both receptors is rapidly induced by pheromone stimulation, and in both cases receptor phosphorylation is necessary for the subsequent ubiquitination step.[64] Convincing evidence indicates that monoubiquitination plays a crucial role in mediating internalization of pheromone receptors. First, blocking ubiquitination of the receptor, either via disruption of ubiquitin-conjugating enzymes or via a point mutation at the ubiquitination site, effectively eliminates ligand-induced internalization.[16] More importantly, internalization of the ubiquitination site mutant $Ste2^{K337R}$ can be restored via an in-frame fusion of ubiquitin to the receptor.[65] Therefore, ubiquitination is both necessary and sufficient to induce internalization of pheromone receptors from the cell surface. In addition to promoting receptor internalization, ubiquitination also serves as a signal to sort the receptors into multivesicular bodies of the late endosome for delivery into the vacuole for degradation.[48]

The molecular basis for both the internalization and the sorting function of ubiquitin has now become clear. It turns out that many proteins involved in endocytosis carry distinct sequence motifs that bind directly to monoubiquitin.[42,48] A number of so-called ubiquitin-binding domains or motifs have been characterized, which include UBA (UBiquitin Associated),[66] UIM (Ubiquitin-Interacting Motif),[67] UEV (Ubiquitin conjugating enzyme E2 Variant),[68] and CUE (Coupling of Ubiquitin conjugation to ER degradation), or NZF (Npl4 Zinc Finger) domains.[69,70] The surfaces on ubiquitin that interact with those domains are overlapping and all share a similar hydrophobic patch that surrounds the residue Ile44.[42] At the internalization step, the adaptor proteins that link ubiquitinated receptors and the endocytosis machinery appear to be epsins (Eps15-interacting proteins) and Eps15 (epidermal growth factor receptor substrate 15, with a yeast homologue Ede1).[71,72] Epsins are ubiquitin-binding domain-containing proteins that function in the assembly of clathrin-coated endocytic vesicles at the cell surface. The adaptor protein required for the sorting of ubiquitinated receptors to the endosome and lysosome appears to be Vps27, a protein carrying UIM domains that bind ubiquitin directly.[72,73]

The findings described above for pheromone receptors were the first to demonstrate that ubiquitination can serve as a signal for inducing endocytosis and the subsequent degradation of an integral membrane protein. These findings also revealed 2 general features of the receptor degradation pathway that are distinct from those used by most cytoplasmic substrates. First, receptors are usually modified by a single ubiquitin molecule (ie, they undergo monoubiquitination), whereas most cytoplasmic substrates are modified by a chain of ubiquitin molecules (undergo polyubiquitination); second, monoubiquitinated receptors are typically delivered via the endocytotic pathway to the vacuole for degradation, whereas polyubiquitinated cytoplasmic proteins are rapidly captured and destroyed by the proteasome. One significant remaining question is what factors prevent the receptors from becoming polyubiquitinated. It is unlikely to be attributable to distinct ubiquitin ligases used, because the ligase Rsp5 responsible for the ubiquitination of pheromone receptor[74] can catalyze both mono- and polyubiquitination of other substrates.[75] One possibility is that once the receptor becomes modified by a single copy of ubiquitin, the modified form is quickly recognized by adaptor proteins such as Epsins, and this binding precludes additional rounds of ubiquitination.

Subsequent to the discovery of pheromone receptor ubiquitination, a series of GPCRs in mammalian cells were also found to undergo stimulus-dependent ubiquitination. So far, the best understood examples are the β_2-adrenergic receptor (β_2-AR) and the chemokine receptor CXCR4.[18,19,76,77] Agonist stimulation triggers phosphorylation of β_2-AR and the recruitment of β-arrestin, an adapter protein known to be essential for the subsequent endocytosis and downregulation of activated receptor.[78] A seminal work by Shenoy, Lefkowitz, and colleagues demonstrates that agonist binding triggers rapid ubiquitination of both the β_2-AR and β-arrestin.[18] Somewhat different from the situation in yeast, ubiquitination does not appear to be necessary for the internalization of the β_2-AR, as a lysine-less mutant does not undergo ubiquitination yet still internalizes normally.[18] However, similar to pheromone receptors, ubiquitination of the β_2-AR promotes its degradation, as the lysine-less β_2-AR displays much impaired degradation.[18] Interestingly, internalization of β_2-AR requires that β-arrestin is also ubiquitinated, as blocking the activity of the E3 ubiquitin ligase for β-arrestin effectively abolishes receptor internalization.[18] Given that there is no obvious β-arrestin homolog in yeast and that β_2-AR and β-arrestin exist as a complex, ubiquitination of β-arrestin appears to provide a *trans* signal for inducing the internalization of the receptor, whereas ubiquitination of the yeast pheromone receptor provides a *cis* signal for its own internalization.

Another well-characterized GPCR that undergoes ubiquitination is the chemokine receptor CXCR4. Similar to the case of β_2-AR, stimulus-dependent internalization of the receptor is not dependent on its ubiquitination.[19] In this case, the internalization signal is composed of a di-Leu motif and conserved Ser residues. Similar to β_2-AR as well as to the yeast pheromone receptors, delivery and degradation of CXCR4 at the lysosome is dependent on ubiquitination.[19,79] Taken together, it appears that the function of ubiquitination for sorting internalized GPCRs for their lysosomal degradation is highly conserved from yeast to humans.

Ubiquitination and Destruction of Heterotrimeric G-Protein Subunits: An Additional Mechanism for Turning Off G Protein Activity

Heterotrimeric G proteins are membrane associated proteins that directly transmit signals from cell-surface receptors to intracellular downstream effectors. The position of G proteins in the signaling pathway is well suited for regulation. Indeed the activation status of heterotrimeric G proteins is a major determinant of signaling strength and duration.[80,81] One major

mechanism to ensure transient signaling by G proteins is via the actions of regulators of G-protein signaling (RGS proteins), a family of proteins that turn off the G-protein activity by accelerating the GTPase activity of Gα.[81,82] Accumulating evidence indicate that heterotrimeric G proteins are also subject to regulation via ubiquitination and degradation.

The first hint that G proteins are regulated by ubiquitination came from studies of the yeast Gα protein Gpa1. Gpa1 is required for adaptation to pheromone stimulation by sequestering the βγ subunits Ste4/Ste18[83] (Figure 2). Gpa1 was shown to be ubiquitinated more than a decade ago in a study designed to identify substrates for the then-newly discovered N-end rule pathway,[21] which relates the in vivo half-life of a protein to the identity of its N-terminal residue.[84] Ubiquitination and degradation of Gpa1 was observed when the N-end rule pathway components, such as the E3 ligase Ubr1, were overexpressed in yeast cells.[21] To explore the physiological relevance of Gpa1 ubiquitination, a proteomic approach was performed to identify the in vivo ubiquitination site(s) on Gpa1.[85] Modification by ubiquitin can be determined by mass spectrometry because trypsin cleavage of isopeptide-linked ubiquitin at the junction between Arg74 and Gly75 would leave the C-terminal 2 amino acids (−Gly-Gly) of ubiquitin attached to the substrate (corresponding to an increase in mass of 115 Da). As ubiquitinated proteins are generally present in low abundance and are intrinsically unstable, an approach of enriching and stabilizing ubiquitinated Gpa1 was devised, most notably by coexpressing a protease resistant N-terminally myc-tagged variant of ubiquitin in cells[86] and purifying the ubiquitinated Gpa1 under strong denaturing conditions. Tandem mass spectrometry analysis of the ubiquitinated peptides identified Lys165 as an in vivo ubiquitination site for Gpa1. A point mutation at the identified ubiquitination site (ie, $Gpa1^{K165R}$) resulted in a substantial decrease in Gpa1 ubiquitination and a diminished ability to respond to pheromone stimulation.[85] These findings indicated that ubiquitination indeed occurs at Lys165, and this modification is important for pheromone signaling. Lys165 lies within the helical domain of the Gα, a region of previously unknown function[85]; thus this work also provided initial evidence that the helical domain is involved in the regulation of Gα stability.

As noted above, ubiquitinated substrates are modified either with a single moiety of ubiquitin (monoubiquitination) or with a chain of ubiquitin (polyubiquitination).[37] Interestingly, Gpa1 can undergo both mono- and polyubiquitination.[87] Moreover, under conditions that accumulate monoubiquitinated Gpa1, the protein becomes enriched in the lysosome compartment, whereas for polyubiquitinated Gpa1, the protein is largely associated with the proteasome; in a mutant strain that accumulates both mono- and polyubiquitinated Gpa1, both lysosomal and proteasomal localization of the protein is evident.[87] Therefore the main function of ubiquitination appears to promote the removal of Gpa1 from the plasma membrane, and the extent of ubiquitination appears to dictate the intracellular destination of the protein. Consistent with this notion, a Gpa1 mutant that does not undergo ubiquitination at all is localized exclusively at the plasma membrane and displays an enhanced ability to suppress signaling mediated by the βγ subunits.[87]

The roles of these 2 seemingly separate pathways for Gpa1 destruction are unclear. One possibility is that Gpa1 is polyubiquitinated when misfolded, misassembled, or mislocalized. Indeed it has been estimated that as much as 30% of newly synthesized proteins are incorrectly made and quickly degraded.[88] Conversely, Gpa1 monoubiquitination may occur as part of the normal protein-clearance process. Stated differently, polyubiquitination could represent a mechanism for quality control, whereas monoubiquitination places a time limit on the lifetime of the functional protein.

An arising issue is whether ubiquitination contributes to a newly discovered endosomal signaling function of Gpa1.[89] Traditionally, it was thought that the main function of Gpa1 in the pheromone response pathway is to sequester plasma membrane–associated free βγ, which activates downstream effectors including components of the MAPK cascade.[80] However, a recent study indicates that a portion of Gpa1 is also localized to the endosome and activates the endosomal phosphatidylinositol 3-kinase Vps34.[89] It is possible that monoubiquitination helps to deliver Gpa1 to the endosome, where it can carry out this second signaling function.[90] In this scenario, ubiquitination and endocytosis of Gpa1 might alter signaling output, by redirecting Gpa1 from the plasma membrane to the endosome. A detailed temporal and spatial analysis of Gpa1 ubiquitination in response to pheromone stimulation would be essential for answering these questions.

The analysis of Gpa1 ubiquitination benefited greatly from several unique features of the yeast experimental system. First, it is relatively easy to obtain large quantities of purified protein, through large-scale fermentation and affinity tag purification. Second, a variety of genetic tools can be used to retard the destruction of newly ubiquitinated substrates, including proteasomal protease mutants and protease-resistant variants of ubiquitin.[91] In fact, Gpa1 represents the first example of direct identification of an in vivo ubiquitination site of any protein using mass spectrometry. This crucial information allowed the rational design of a mutant form of Gpa1 that does not undergo ubiquitination. Another unique feature of the yeast system is the ability to manipulate the extent of ubiquitination through the use of mutants that selectively stabilize the mono- or polyubiquitinated substrates. Myc-ubiquitinated proteins are poor substrates for proteolysis and, therefore, accumulate to higher-than-normal levels within the cell.[86] *pep4* mutants disrupt vacuolar protease function and have been successfully used to enrich monoubiquitinated substrates including Gpa1.[16] *cim3* mutants disable proteasome protease activity and, therefore, result in the accumulation of polyubiquitinated substrates including Gpa1.[92] Although less commonly applied, *ubp* mutants lacking ubiquitin processing proteases can also be very effective in preserving short-lived changes in protein ubiquitination.[23,87] This approach is analogous to using specific phosphatase mutants or inhibitors to preserve transient increases in protein phosphorylation.

In mammalian cells, heterotrimeric G-protein subunits have been demonstrated to be regulated by the proteasomal degradation pathway. Well-documented examples include

$G\alpha_o$,[27] $G\alpha_{i3}$,[28] $G\alpha_{i2}$,[93] $G\alpha_s$,[26] $G\gamma_2$,[25] and transducin $G\beta\gamma$.[24] Among those, direct ubiquitination has been observed for $G\alpha_s$, $G\gamma_2$, and transducin $G\gamma$, whereas the others were mainly inferred from studies showing diminished destruction by specific inhibitors of the proteasome. In any event, it appears that regulated destruction of heterotrimeric G-protein subunits is a highly prevalent phenomenon, and likely represents a common mechanism for turning off G-protein activity.

Ubiquitination and Destruction of RGS Proteins: Resensitizing the Pathway

RGS proteins are a family of regulatory proteins whose main function is to switch off the activity of heterotrimeric G proteins and do so by accelerating the GTPase activity of $G\alpha$ and thereby promoting the reassociation of the G-protein subunits.[82] The first identified RGS protein was the yeast Sst2 protein, which is responsible for promoting pheromone desensitization by accelerating the GTPase activity of Gpa1.[94] Interestingly, recent work from both yeast and mammalian systems provide good evidence that RGS proteins are subject to regulation by the ubiquitination pathway.

Ubiquitination and destruction of the yeast RGS protein Sst2 was discovered in a recent computational modeling analysis of the dynamic changes in signal propagation along the yeast pheromone pathway.[22] In that analysis, the amount of the RGS and G protein in living cells, as well as the changes in their abundance in response to pathway activation, were measured. A model based on ordinary differential equations was able to confirm the existence of a negative feedback loop in which increased synthesis of the RGS protein leads to signal inactivation. However, an inconsistency with the computational and experimental data suggested the existence of an additional, previously unsuspected, positive-feedback loop. In testing the revised model the RGS protein Sst2 was predicted to undergo stimulus-dependent degradation. In confirmation of the model, Sst2 was found to undergo stimulus-dependent ubiquitination and degradation.[22] Thus, the stimulus-dependent synthesis of RGS proteins leads to feedback inhibition of G-protein signaling, whereas a later stage of stimulus-dependent ubiquitination and destruction of RGS proteins leads to resensitization of the pathway.

The molecular mechanism underlying Sst2 ubiquitination has not been fully characterized. Preliminary results suggest that the ubiquitin ligase Ubc2/Ubr1 might be responsible for Sst2 ubiquitination, as Sst2 ubiquitination and degradation is dramatically decreased in yeast strains that are defective in either *UBC2* or *UBR1* expression.[22] Ubc2 is an E2 enzyme that acts in concert with the E3 ligase Ubr1 to target N-end rule substrates for degradation.[21] As mentioned earlier, another well characterized N-end rule substrate in the pathway is the yeast $G\alpha$ Gpa1. As Gpa1 and Sst2 are both functionally and physically linked in the pathway,[95] it would be interesting to determine whether these 2 proteins are targeted by the same ligase, either as a complex or individually, leading to coordinated regulation of both proteins.

In mammalian cells, strong evidence indicates that the cellular concentrations of several RGS proteins including RGS4, RGS5, and RGS16 are likewise regulated by N-end rule pathway.[96] RGS4 was initially identified as a substrate of the N-end rule pathway in a reticulocyte lysate expression system.[30] Recognition of RGS4 by the N-end rule pathway ligases Ubr1 and/or Ubr2 requires the enzymatic conjugation of an arginine to its N terminus.[29] This reaction is catalyzed by Arg/transfer RNA protein transferase and occurs on the cysteine 2 at the N terminus of RGS4. Interestingly, mouse arginine transferase knockout embryos are impaired in G_q and G_i signaling, accumulate high levels of RGS4 and RGS5, and die with defects in heart development and angiogenesis.[29,31] As G_q- and G_i-mediated signaling are negatively regulated by RGS4 and RGS5,[97–99] the cardiovascular phenotypes are consistent with the notion that appropriate cellular concentration of RGS proteins is important for proper cardiovascular signaling. Whether or not other components essential for cardiovascular signaling are targeted by the arginylation-dependent N-end rule pathway remains to be determined.

Ubiquitination of MAPK Cascade Components: For Destruction and More

GPCRs are commonly coupled to the activation of mitogen-activated protein kinase (MAPK) cascades.[1] The best understood mechanism for the regulation of MAPK signaling is undoubtedly the phosphorylation of various components in the cascade.[4] Typically, each kinase in the cascade is activated via phosphorylation by an upstream kinase and is inactivated via dephosphorylation by relevant phosphatase(s). Accumulating evidence from both yeast and mammalian cells indicate that certain components of the MAPK cascade also undergo ubiquitination.[100] For the majority of substrates reported so far, the main role of ubiquitination appears to be permanent inactivation of the components that are targeted for destruction.[100] However, there are instances in which ubiquitination of certain components of the MAPK cascade does not lead to protein degradation, but rather appears to have a direct role in regulating the activity and/or localization of the substrates.[33,101] More detailed analysis of these processes would likely reveal novel mechanisms for the regulation of MAPK signaling.

In yeast the MEK Ste7 is the only component in the pheromone signaling pathway that has been convincingly demonstrated to undergo stimulus-dependent ubiquitination.[23] Earlier work suggests that Ste7 might be a limiting component in the cascade, because it is expressed at much lower levels than either the upstream kinase Ste11 or its downstream targets, the MAPKs Fus3 and Kss1.[102,103] Ubiquitination and degradation of Ste7 requires a functional Skp1/Cullin/F-box (SCF) complex,[104] suggesting SCF might be the ligase for Ste7. Indeed, similar to many SCF substrates, Ste7 is ubiquitinated only after it has become phosphorylated by its upstream kinase Ste11.[104] Therefore phosphorylation of Ste7 by Ste11 seems to serve 2 functions: activation as well as the subsequent destruction of Ste7, thereby limiting the active lifetime of Ste7. SCF ubiquitin ligase was previously shown to ubiquitinate components of the cell cycle machinery during the G_1-to-S transition.[39,105] Ste7 is well known to mediate pheromone-induced cell-division arrest at G_1.[80] Therefore it appears that SCF might promote cell-cycle progression through coordinated regulation of the cell-cycle

machinery as well as the Ste7 signaling pathway that leads to cell-cycle arrest.

Besides Ste7, the only other component in the cascade shown to be degraded on pheromone stimulation is its upstream kinase Ste11.[106] Although pheromone-induced destruction of Ste11 is clearly dependent on a functional DUB Ubp4, a direct demonstration of Ste11 ubiquitination is lacking. This could be attributable to the inherent difficulty associated with the detection of ubiquitinated species, which are usually extremely unstable.[107] As Ste7 and Ste11 are normally part of the same signaling complex, an alternative possibility is that degradation of Ste11 is somehow dependent on the ubiquitination of Ste7.

Another MEK that has been demonstrated to undergo ubiquitination is MEK1 in *D discoideum*.[108] Similar to Ste7, ubiquitination of MEK1 is elevated after pathway stimulation. The ligase that ubiquitinates MEK1 appears to be MIP1, a MEK1-interacting protein with a RING finger domain at its C terminus, as recombinant MIP1 protein possesses ligase activity in vitro and ubiquitination of MEK1 is blocked in *mip1*-null cells in vivo[108]. In addition, in vivo interaction of MEK1 and MIP1 is enhanced by chemoattractant stimulation, and the kinetics of their enhanced interaction is coincident with that of MEK1 ubiquitination.[108] Interestingly, despite the fact that MEK1 ubiquitination is stimulus dependent, ubiquitination per se does not require MEK1 activation, as a nonactivating form of MEK1 is still ubiquitinated with kinetics similar to that of the wild-type protein.[108] Thus other mechanisms following pathway activation must exist to target MEK1 for ubiquitination. One possibility is that the ligase MIP1 is regulated by kinase(s) upstream of MEK1 in the pathway.

In mammalian cells, known ubiquitination substrates include MEKK1 and several isoforms of MAPKs (extracellular signal-regulated kinases [ERKs] 1, 2, 3, and 7).[100] MEKK1 is a MEKK that phosphorylates and activates MEKs MKK1 and MKK4, which in turn activates the MAPKs ERK1/2 and c-Jun N-terminal kinase (JNK).[2] MEKK1 has a C-terminal protein kinase domain and a unique N-terminal RING finger-like PHD, which possesses E3 ubiquitin ligase activity.[34] The E3 activity of MEKK1 requires the kinase activity of the full-length MEKK1 protein.[33,34] Interestingly, the downstream MAPKs ERK1 and ERK2 have been demonstrated to be in vivo ubiquitination substrates of MEKK1.[34] Ubiquitination of ERK1/2 by MEKK1 appears to target them for degradation in the proteasome.[34] Therefore, MEKK1 acts not only as an upstream activator of ERK1, ERK2, and JNK via its kinase domain but also as an inactivator of ERK1 and ERK2 via its E3 ubiquitin ligase activity. MEKK1 also targets itself for ubiquitination.[33] Remarkably, in this instance, ubiquitination does not lead to MEKK1 degradation, but rather inhibits its catalytic activity as a protein kinase. Consequently, activation-dependent autoubiquitination of MEKK1 provides a novel mechanism for inhibiting MEKK1-catalyzed phosphorylation of MKK1 and MKK4, thereby downregulating the activation of ERK1/2 and JNK pathways mediated by MEKK1.

MAPKs demonstrated to undergo ubiquitination include ERK1 and ERK2, 2 classical MAPKs that consist mainly of a kinase domain. Ubiquitination and degradation of ERK1 and ERK2 has so far only been shown to occur on stress stimulation such as sorbitol treatment, and this reaction requires the E3 ligase activity of their upstream kinase MEKK1,[34] as discussed above. A potential physiological function of stress- and MEKK1-induced ubiquitination and degradation of ERK1 and ERK2 could be to promote apoptosis, by downregulating survival signaling mediated by ERK1/2. Indeed, blocking ubiquitination of ERK2 via disruption of its interaction with the ligase MEKK1 reduces sorbitol-induced apoptosis.[34] In addition to ERK1/2, ERK1c, an alternatively spliced form of ERK1, also undergoes ubiquitination.[101] ERK1c contains an insertion of 103 base pairs that possesses a stop codon at positions 55 to 57 of the insert, which results in a substitution of the last 40 aa of ERK1 with 18 aa of the insert.[101] Whereas ERK1 and ERK2 undergo polyubiquitination, ERK1c appears to be mainly regulated by monoubiquitination.[101] Interestingly, monoubiquitination of ERK1c correlates nicely with its localization at the Golgi apparatus. In cells grown to high density, both monoubiquitination and Golgi accumulation of ERK1c are increased, whereas in low-density cells, accumulation of monoubiquitinated ERK1c at the Golgi is seen only after addition of the proteasome inhibitor MG132.[101] However, it is unclear why MG132 elicits such an effect on ERK1c monoubiquitination as well as its localization.

Other ERK isoforms (ie, ERK3 and ERK7) that are regulated by ubiquitination and degradation belong to the so called "big" MAPKs, which have a large C-terminal extension in addition to their N-terminal kinase domain. Distinct from ERK1/2, both ERK3 and ERK7 are unstable proteins and are expressed at low levels.[35,36] Moreover, ubiquitination and degradation of ERK3 does not require any internal lysine residues in the protein. Rather, the free amino group of its N-terminal residue is used for conjugation of the ubiquitin chain.[109] In proliferating cells, ERK3 is rapidly and constitutively degraded in a manner that does not require its kinase activity. During muscle differentiation, the stability of ERK3 is dramatically increased.[35] Therefore, proper progression of the cell cycle appears to correlate with disappearance of ERK3, which suggests that ERK3 may have some inhibitory effects on cell-cycle progression. Consistent with this notion, expression of stabilized forms of ERK3 inhibit entry into S phase.[35] Similar to ERK3, ubiquitination and degradation of ERK7 also depends on its N-terminal region.[36] Interestingly, the 20-aa N terminal appears to be both necessary and sufficient to confer instability, as fusion of this region can cause degradation of other heterologous proteins such as ERK2 and green fluorescent protein.[36]

Implications for Cardiovascular Disease and Its Treatment

Appropriate signaling by G protein– and MAPK-mediated pathways is critical for maintaining normal function in the cardiovascular system.[110–112] In addition, activation of certain MAPK family members such as the ERKs and p38 appears to be partially responsible for the pathogenesis of various processes in the heart, including myocardial hypertrophy, heart failure, ischemia/reperfusion injury, and cardioprotec-

tive responses.[111] As such, G protein– and MAPK-mediated signaling pathways are major targets for the pharmacological treatment of cardiovascular disease. Indeed, the development of β blockers, which specifically antagonize the signaling by β-ARs, has revolutionized the medical management of hypertension and coronary artery disease. Drugs of this type were the prototype for all receptor antagonists, which are now widely used in clinical pharmacology.

The new findings that multiple steps of G protein- and MAPK-mediated signaling pathway are regulated via ubiquitination will surely reveal new mechanisms of cell regulation and eventually open new avenues for the treatment of cardiovascular diseases. For instance, misregulation of specific factors required for protein ubiquitination, such as adaptors, ligases, or DUBs, could contribute to inappropriate activation of G protein– and MAPK signaling. By the same token, given that distinct isoforms of G proteins and MAPKs are differentially regulated by ubiquitination, drugs targeting specific ubiquitinating or DUBs could selectively activate or inhibit their signaling activities. Just as protein phosphatase inhibitors can be used to stimulate the activation of certain MAPKs to exert cardioprotection effects,[113] it is highly likely that small molecule inhibitors of DUBs could be developed to serve a similar purpose.

Conclusions and Future Perspectives

A large array of cellular processes are controlled by signaling pathways mediated by G proteins and MAPKs. Thus it is not surprising that those signaling proteins are under intensive regulation. In addition to extensively studied mechanisms such as phosphorylation, ubiquitination has emerged as a central regulator of signaling cascades. As outlined in this article, many key components in the pathway are regulated by ubiquitination. In most instances, ubiquitination is triggered by pathway activation and acts as a negative feedback mechanism to destroy signaling components, dampening the signal and thereby resetting the pathway to pre-stimulatory status. Apart from regulating the stability of signaling components, ubiquitination is also able to modulate the subcellular localization and, in some instances, even the activity of signaling components. Given the prevalence of signaling proteins shown to undergo ubiquitination and the functional versatility of this posttranslational modification, this will surely be a topic of intense investigation in future years.

Despite tremendous progress made in understanding the regulation of G protein– and MAPK-mediated signaling pathways by ubiquitination, many challenges remain. Additional ubiquitination substrates in these pathways are likely to exist, and effective strategies for their identification need to be developed. In this regard, genetic approaches to systematically inactivate deubiquitinating enzymes would likely help, as exemplified by the use of ubp3 mutants in the discovery of Ste7 ubiquitination. Whereas past studies have examined temporal aspects of protein ubiquitination, our understanding of spatial aspects of regulation is lacking. Given that most signaling proteins reside in multiple subcellular locations, and exert quite distinct functions in each location, understanding where and how ubiquitination occurs is needed to fully understand the function of the modification. As demonstrated by GPCRs as well as ERK1c, it is becoming increasingly apparent that ubiquitination might play important roles in targeting signaling proteins to distinct locations and, in this manner, to regulate signaling specificity. However, in those instances and particularly for the case of ERK1c, the downstream events required to achieve proper targeting of ubiquitinated species has not been fully elucidated. A proteomic approach to enrich and identify the factors that specifically recognize monoubiquitinated GPCRs as well as ERK1c would be tremendously valuable in this regard. Finally, investigations of signaling protein ubiquitination commonly consider only the behavior of individual substrates. How those individual ubiquitination events contribute to the behavior of signaling networks is poorly understood. In this regard, a combination of experimental approaches and mathematic modeling of network behavior might prove to be a valuable strategy. Yeast has already proven to be a powerful tool in elucidating fundamental principles of pathway regulation, and the lessons learned from this simple model system will no doubt continue to guide parallel studies in more complex cell systems in the future.

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Disclosures

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