Molecular Pharmacology Fast Forward. Published on July 27, 2010 as doi:10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Thinking outside of the (RGS) box: New Approaches to Therapeutic Targeting of Regulators of G protein Signaling

Benita Sjögren and Richard R. Neubig

Department of Pharmacology (BS, RRN) and Department of Internal Medicine (Cardiovascular Medicine) (RRN) University of Michigan, MSRB III, Ann Arbor, MI 48109

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Approaches to targeting of RGS proteins in drug discovery

Corresponding author: Richard R. Neubig

University of Michigan,

1150 W Medical Center Dr,

MSRB III,

Ann Arbor, MI 48109

Phone: +1-734-763-3650

Fax: +1-734-763-4450

e-mail: rneubig@umich.edu

Text pages: 14

Figures: 1

References: 72

Words in abstract: 180

Words in introduction: 449

Abbreviations: GPCR, G protein-coupled receptor; RGS, Regulator of G protein signaling; GAP, GTPase activating protein; AC, Adenylate cyclase; PLCβ, Phospholipase Cβ; DEP, Disheveled-EGL10-pleckstrin; R7BP, R7 binding protein; R9AP, RGS9 anchoring protein; GGL, Gγ-like; GDI, Guanine nucleotide dissociation inhibitor; LC, Locus coeruleus; NAc, Nucleus accumbens

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Regulators of G protein signaling (RGS) proteins are emerging as potentially important drug targets. The mammalian RGS protein family has more than 20 members and they share a common ~120 residue RGS homology (RH) domain or "RGS box". RGS proteins regulate signaling via G protein-coupled receptors (GPCRs) by accelerating GTPase activity at active α subunits of G proteins of the Gq and Gi/o families. Most studies searching for modulators of RGS protein function have been focused on inhibiting the GTPase accelerating protein (GAP) activity. However, many RGS proteins contain additional domains that serve other functions such as interactions with proteins or subcellular targeting. Here, we discuss a rationale for therapeutic targeting of RGS proteins by regulation of expression or allosteric modulation to permit either increases or decreases in RGS function. Several RGS proteins have reduced expression or function in pathophysiological states so strategies to increase overall RGS function would be useful. Since several RGS proteins are rapidly degraded by the N-end rule pathway, finding ways to stabilize them may prove to be an effective way to enhance RGS protein function.

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

G protein-coupled receptors (GPCRs) represent one of the largest groups of proteins with over 1,000 members. They mediate key physiological responses and their endogenous agonists include small molecule neurotransmitters, hormones, peptides, and for some receptors even their own N-terminus. The importance of GPCRs in therapeutics is demonstrated by the fact that about 30% of FDA approved drugs target these receptors (Williams and Hill, 2009). GPCRs couple to heterotrimeric G proteins consisting of an α and a βγ subunit. In its inactive state, the α subunit is bound to GDP. Upon agonist binding to the receptor the G protein gets activated and the GDP is exchanged for GTP; the α and βγ subunits dissociate and both can mediate downstream signaling. The signal is turned off when the GTP is hydrolyzed back to GDP. G proteins have built-in GTPase activity however this process is very slow and does not correspond to the rapid turnoff of signaling events in cells. Regulators of G protein signaling (RGS) proteins have emerged in the last 15 years as important intracellular regulators of GPCR signaling (see Hollinger and Hepler, 2002; Ross and Wilkie, 2000; Zhong and Neubig, 2001 for review). They reduce the amplitude and duration of G protein signaling by binding to activated Gα subunits and dramatically increasing their GTPase activity. This GTPase accelerating protein (GAP) activity of RGS proteins is largely limited to α subunits of the Gq and Gi/o families of G proteins. There are no widely accepted reports of RGS proteins acting on Gs although it has been suggested that RGS proteins can indirectly regulate $G\alpha_s$ signaling via interactions with certain adenylate cyclase subtypes (Roy et al., 2006; Talbot et al., 2010).

The structure and function of RGS proteins (Hollinger and Hepler, 2002) and implications for them as drug targets (see e.g. Neubig and Siderovski, 2002; Ross and Wilkie, 2000; Traynor and Neubig, 2005; Zhong and Neubig, 2001) have been extensively reviewed previously. The main focus of efforts to identify modulators of RGS protein function has thus far been targeted towards inhibiting the GAP activity and/or the interaction with Gα subunits. However many RGS proteins display other functions, including protein-protein interactions

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

and regulation of cellular trafficking. This indicates that inhibiting RGS protein function might not be as simple as inhibiting GAP activity towards Gα subunits. Recent data also suggest that RGS protein expression may be altered in certain pathophysiological states and several RGS proteins have been shown to be rapidly degraded in cells. This review explores the possibility of pharmacologically modulating RGS protein expression as an alternative route to alter RGS protein function. Furthermore we discuss possible ways to allosterically modulate RGS protein function through other mechanisms than competitively inhibiting GAP activity.

RGS proteins – more than just GAPs on Gα

There are 20 known mammalian RGS proteins which have strong GAP activity at Gα subunits and they are divided into several subfamilies based on sequence homology (R4, R7, R12, and RZ). Their structure and function have been extensively reviewed elsewhere so those aspects will not be discussed in detail here (see (Hollinger and Hepler, 2002; Ross and Wilkie, 2000). In addition to these four "classical" RGS families, there are a number of other proteins with RGS homology (RH) domains (Tesmer, 2010) which will not be covered in this review. Many RGS proteins have additional domains apart from the RGS domain (e.g. Hollinger and Hepler, 2002; Neubig and Siderovski, 2002; Ross and Wilkie, 2000). This suggests that RGS proteins can have other functions than just accelerating GTP hydrolysis (see below). Coincidently, a recent review provided a detailed overview of non-GAP functions of RGS proteins, including regulation of receptor tyrosine kinase signaling and nuclear functions (Sethakorn et al., 2010). Given these additional functions, targeting these proteins in drug discovery may not be as simple as just inhibiting GAP activity.

With the exception of RGS3, the R4 family members (RGS 1-5, 8, 13, 16, 18 and 21) are small structures with only minimal C- and N-terminal extensions flanking the RGS domain.

RGS3 exists as three splice variants and the two longer forms, RGS3L and PDZ-RGS3 contain domains involved in protein-protein interactions (Kehrl et al., 2002). Despite the apparent lack of additional structured domains, several R4 family members have been shown to possess functions beyond GAP activity (reviewed in Bansal et al., 2007). The N-terminus of R4 family RGS proteins contains an amphiphatic α-helix that serves as a membrane targeting signal. This targeting is necessary for proper function of the proteins as it brings them close to the site of action i.e. activated Gα subunits (Bernstein et al., 2000; Saitoh et al., 2001). Furthermore, several members of the R4 family have been shown to interact with components of GPCR signaling, such as receptors, adenylate cyclase (AC) subtypes and phospholipase Cβ (PLCβ) (reviewed in Bansal et al., 2007). This, in addition to different affinities for Gα protein subtypes, contributes to selectivity among R4 family RGS proteins. Most members are relatively promiscuous for Gi/o and Gq proteins but RGS2 has been shown to be selective for Gq (Heximer et al., 1997). Although there are no widely accepted reports on RGS proteins with GAP activity towards $G\alpha_s$ proteins there have been reports of RGS2 regulating $G\alpha_s$-mediated cAMP signaling through a direct protein-protein interaction with certain AC subtypes (ACII, IV and VI) (Roy et al., 2006).

The members of the R7 family of RGS proteins (RGS 6, 7, 9 and 11) contain a disheveled-EGL10-Pleckstrin homology (DEP) domain that is responsible for protein-protein interactions. Specifically, this domain serves as an interaction site with R7 binding protein (R7BP) in the brain and RGS9 anchoring protein (R9AP) in the retina to anchor the RGS protein to the plasma membrane near its site of action (Drenan et al., 2006; Jayaraman et al., 2009). The DEP domain can also interact with receptors to directly modulate GPCR signaling (Ballon et al., 2006). The R7 family members also contain a Gγ-like (GGL) domain which binds $G\beta_5$, an interaction that is necessary for stable expression of both proteins. $G\beta_5$ knock-out mice also lack expression of all four R7 family RGS proteins (Chen et al., 2003). Specific

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

targeting of the DEP- or GGL-domain of an R7 family RGS could serve as an alternative route to target function of these proteins. Inhibition of the RGS-$G\beta_5$ interaction would downregulate expression of that particular RGS protein. Similarly, inhibition of the interaction between the DEP domain of RGS9 and R7BP could downregulate RGS9 expression since a couple reports have shown that RGS9 depends on this interaction for stable expression (Anderson et al., 2007a; Anderson et al., 2007b).

The R12 family members RGS12 and 14 also contain additional functional domains. The C-terminal $G\alpha_{i/o}$-Loco (GoLoco) motif has guanine nucleotide dissociation inhibitor (GDI) activity towards $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ (Kimple et al., 2001; Siderovski and Willard, 2005). Binding of the GoLoco motif to Gα inhibits the activation of the G protein by preventing exchange of GDP for GTP. It also blocks association of Gα with Gβγ potentially leading to prolonged βγ signaling.

The members of the RZ family of RGS proteins, which are less well characterized than the other families, all have an N-terminal cysteine string motif (reviewed in Nunn et al., 2006), that is a target for palmitoylation. The cysteine string can serve as a site for protein-protein interactions and the palmitoylation could be a signal for subcellular trafficking or protein stability (reviewed in Linder and Deschenes, 2007).

RGS proteins are important targets for drug discovery

Since their discovery about 15 years ago, important biological roles for RGS proteins have been identified. Given their role in regulating GPCR signaling it is not surprising that they have been suggested to be potentially interesting targets for drug discovery. The literature on the physiological functions of RGS proteins has expanded greatly in recent years so only selected aspects of RGS protein function will be discussed here (for more extensive reviews see e.g Blazer and Neubig, 2009; Hepler, 1999; Hollinger and Hepler, 2002; Ross and Wilkie,

2000; Sjögren et al., 2010; Traynor and Neubig, 2005; Zhong and Neubig, 2001). The numerous RGS knock-out mouse models have been reviewed recently (Kaur et al., 2010).

Substantial data demonstrate roles for endogenous RGS proteins in cardiovascular functions, such as regulation of blood pressure and cardiac rhytmicity. RGS2 is expressed in vascular smooth muscle cells and heart and RGS2 knock-out mice are hypertensive, suggesting a role in blood pressure homeostasis (Heximer et al., 2003). In contrast, RGS5 knock-out mice were shown in one study to be hypotensive (Cho et al., 2008), possibly suggesting a tightly regulated balance between different RGS proteins in the control of physiological responses. RGS proteins have also been demonstrated, by us and others, to regulate cardiac rhythmicity through actions in the sino-atrial and atrio-ventricular nodes in the heart (Bender et al., 2008; Cifelli et al., 2008; Fu et al., 2007; Fu et al., 2006).

There is substantial CNS expression of many RGS protein subtypes (Gold et al., 1997). Several, including RGS4 and RGS9, control effects of opiates and other drugs of abuse (Gold et al., 2003; Rahman et al., 2003; Traynor and Neubig, 2005; Zachariou et al., 2003). RGS4 associates with μ- and/or δ-opioid receptors in vitro and inhibits signaling via these receptors (Georgoussi et al., 2006; Wang et al., 2009b). Although RGS4 knock-out mice initially showed no difference in opiate dependence (Grillet et al., 2005), targeted RGS4 knock-down in the locus coeruleus (LC) increased morphine reward whereas in the nucleus accumbens (NAc) RGS4 acted as a negative regulator of opioid dependence (Han et al., 2009). RGS9 knock-out mice have markedly enhanced opioid-induced analgesia and are more susceptible than wild-type mice to the development of morphine dependence implicating RGS9 as an important regulator of opioid functions in the CNS (Zachariou et al., 2003) along with RGS4.

RGS9 is highly expressed in the striatum and RGS9 knock-out mice have impairments in motor coordination (Blundell et al., 2008). They develop more severe L-DOPA-induced

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

dyskinesias compared to wild-type mice in models of Parkinson's disease (PD) (Gold et al., 2007). In addition, overexpression of RGS9 in the striatum in primate models of PD attenuates L-DOPA-induced dyskinesias (Gold et al., 2007).

Evidence is emerging for the role of RGS proteins in cancer biology. GPCR signaling in cancer progression is an area of increasing interest and thus several RGS proteins have been implicated in regulating GPCR-mediated signals in various cancers (reviewed in Hurst and Hooks, 2009). Altered expression levels of several RGS proteins have been detected in various cancer forms including prostate cancer (RGS2), breast cancer (RGS4) and hepatocellular carcinoma (RGS5). Reduced expression of RGS2 in androgen-independent prostate cancer leads to increased GPCR signaling linked to uninhibited cell growth (Cao et al., 2006). Overexpression of RGS2 in prostate cancer cell lines leads to decreased signaling that is also seen in part with a GAP-deficient mutant, suggesting a mechanism involving non-RGS domains of the protein. A recent study showed that RGS4 is downregulated in breast cancer and overexpression of RGS4 in metastatic breast cancer cells reduced both invasion and migration Xie et al., 2009 and see below).

Given these and many other biological functions attributed to RGS proteins, it is clear that they could serve as important drug targets. Several groups, including our own, have undertaken strategies for high-throughput screening for compounds that modulate RGS protein function. Young and colleagues utilized a yeast two-hybrid approach and identified a series of compounds (Young et al., 2004) and peptides (Wang et al., 2008) that inhibit RGS4 GAP activity by blocking the interaction with $G\alpha_{i2}$. Our lab has developed biochemical methods to screen for inhibitors of the RGS-Gα interaction. The Flow Cytometry Protein Interaction assay (FCPIA) is a bead-based assay that has been adapted to high-throughput screening and can be multiplexed to study the effects of compounds on several RGS proteins in the same well (Roman et al., 2009). Using this method CCG-4986 was identified as an

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

inhibitor of the Gα-RGS4 interaction, with selectivity over other RGS proteins (Roman et al., 2007).

The common denominator of previous studies has been the goal of finding inhibitors of the Gα-RGS interaction. Such compounds would be very useful pharmacological tools to increase understanding of the biological functions of RGS proteins. However, from a clinical perspective, enhancers rather than inhibitors may be more useful in drug development. This is supported by emerging data showing reductions in RGS protein expression or function in several pathophysiological states (see below). Modulators of RGS protein expression could be an effective way to increase (or decrease) the function of these proteins. As discussed above, many RGS proteins have functions besides their GAP activity. Increased expression would also modulate those functions while a RGS/Gα inhibitor would not. An alternative approach to increasing RGS activity, that we will discuss first, is allosteric modulation of RGS protein function.

Allosteric modulation of RGS protein function

The presence of additional domains in many RGS proteins provides mechanisms to allosterically modulate function, both within and from outside of the RGS domain. R7 family RGS proteins form an obligatory dimer with $G\beta_5$ and this interaction is necessary for stable expression. $G\beta_5$ knock-out mice lack expression of all four members of the R7 family of RGS proteins (Chen et al., 2003). Their interaction with the broadly expressed membrane anchor R7BP seems to be more complicated. In most cases this serves to target the RGS proteins to the plasma membrane but in the case of the brain specific isoform of RGS9 it also stabilizes RGS9 protein expression. RGS9, but not RGS7, is rapidly degraded by cysteine proteases in the absence of R7BP (Anderson et al., 2007a; Anderson et al., 2007b).

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Slepak and colleagues (Narayanan et al., 2007; Sandiford and Slepak, 2009) recently reported a novel receptor-specific role of the DEP domain of RGS7 that might be modulated chemically to affect RGS7 function. The RGS7 DEP domain can bind to the 3rd intracellular loop of the M3 (but not the M1) muscarinic receptor and inhibit its coupling to Gq by a mechanism that does not involve the RGS GAP function (Narayanan et al., 2007; Sandiford and Slepak, 2009). In addition, RGS7 interacts with $G\beta_5$ via the GGL domain, as do all members of the R7 family. One study found a novel interaction between the RGS7 DEP domain and recombinant $G\beta_5$ (Narayanan et al., 2007) which was suggested to subserve an intramolecular inhibition role in that the complex. In the intact RGS7/$G\beta_5$ complex a closed form of the DEP/$G\beta_5$ contact was less active at inhibiting $G\alpha_q$ signaling via the muscarinic M3 receptor (Narayanan et al., 2007). Mutations in the RGS7 DEP domain disrupted the $G\beta_5$ interaction suggesting that chemical modulation of that process might lead to increased activity of RGS7 in inhibiting Gq signaling. Perhaps, this could also enhance the DEP-dependent receptor recruitment of R7 family RGS proteins to modulate their classical GAP function as well. Finally, recent crystallographic data suggests that this DEP/$G\beta_5$ contact may also occur for RGS9 (Cheever et al., 2008).

Similar to inhibiting protein-protein interactions involving R7 family RGS proteins and their binding partners, a way to modulate RGS2 function is to inhibit interaction with AC, specifically targeting RGS2 effects on Gs-mediated signaling. Other protein-protein interactions have also been identified that may have potential in drug development. As is discussed in the recent review by Sethakorn et al. (Sethakorn et al., 2010) RGS3 can interact with the Mad homology (MH) domain of Smad3 thereby regulating Transforming growth factor β (TGF-β) signaling (Yau et al., 2008). This interaction was mapped to a region outside the RGS domain and may be another potentially interesting interaction to target. The full

significance of the RGS3-Smad3 interaction is not yet known, but given the role for TGF-β in regulating cell growth and survival this may prove to be show clinically importance.

Phosphorylation of proteins can serve many roles, such as activation or inactivation of an enzyme. RGS16 is phosphorylated at Tyr^{168} in the RGS domain by p60 Src kinase *in vitro* (Derrien et al., 2003). This phosphorylation increased GAP activity and stabilized RGS16 protein expression in stably transfected cells. This could be an important regulatory mechanism for RGS16 activity by dual actions on GAP activity and protein expression and could serve as an alternative route in drug discovery towards RGS16 protein modulators.

Targeting the RGS-Gα interaction has for several years been attempted by us and others. The R4 family of RGS proteins has been extensively studied by and it is the only family of RGS proteins for which small molecule and peptide inhibitors have been reported (Jin et al., 2004; Roman et al., 2009; Roman et al., 2007; Roof et al., 2006; Wang et al., 2008). Despite their small and relatively simple structure, allosteric modulation of GAP activity may be possible. The structure of the RGS domain of RGS4 bound to $G\alpha_{i1}$ (Tesmer et al., 1997) revealed two potential drug binding pockets on the surface of RGS4 and a recent review paper described the structure of RGS domains in detail (Tesmer, 2010). The site of contact between RGS4 and the $G\alpha_{i1}$ subunit in the cocrystal, also known as the A site (Zhong and Neubig, 2001) is a flat surface that may be difficult to target with small molecules. The second potential drug binding pocket is on the ‘‘back’’ of the molecule has been termed the B site (Zhong and Neubig, 2001). This site is more similar in size and shape to ‘‘druggable’’ pockets on other proteins and may serve as a possible allosteric site to modulate GAP activity. Indeed we recently showed that the potent RGS4 inhibitor CCG-4986 binds to two cysteines in the RGS domain of RGS4 (Roman et al., 2010) one of which is located near the proposed B site of the molecule. A second, reversible RGS4 inhibitor also acts on those residues (Blazer et al., 2010).

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

This site also seems to play a role in physiological regulation of R4 family function. Wilkie and co-workers showed that several members of the R4 family of RGS proteins interact with phosphatidyl-3,4,5-trisphosphate (PIP_3) which inhibits GAP activity (Popov et al., 2000). Interestingly, this can be reversed by Ca^{++}/calmodulin binding to a similar site on the RGS domain (Ishii et al., 2005; Ishii et al., 2001; Ishii et al., 2002; Popov et al., 2000). Given the role of R4 family RGS proteins in regulation of $G\alpha_q$ mediated calcium signaling this likely represents an endogenous negative-feedback mechanism whereby Ca^{++} increases RGS activity to suppress Ca^{++} responses. It is intriguing to consider that this natural process could be co-opted pharmacologically to inhibit or enhance R4 family RGS protein function.

RGS proteins can be rapidly degraded

Several RGS proteins have a short *in vivo* half life due to rapid degradation in cells. Furthermore, the expression of several RGS proteins is reduced in various disease states. Ubiquitin-mediated proteasomal degradation is an important regulatory system for many cellular processes including cell cycle control, stress responses, and breakdown of misfolded proteins. These pathways have been well described elsewhere (see e.g.Hershko and Ciechanover, 1998). Briefly, a protein targeted for degradation is coupled to a chain of ubiquitin molecules by a series of enzymes, E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligases). The ubiquitylated protein is then recognized by the 26S proteasome and degraded. Drugs targeting protein degradation are already in use for the treatment of various cancers. The first FDA approved drug was the proteasome inhibitor Bortezomib (Velcade) (reviewed in Adams, 2004). Subsequently, similar drugs, such as Carfilzomib and NPI-0052 have entered clinical trials for the treatment of various cancers (reviewed in Yang et al., 2009). However, targeting general protein degradation will give rise to a number of side effects. A more focused approach could be taken by inhibiting specific

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

enzymes in the degradation pathway. For instance there are more than 500 known mammalian E3 ligases and targeting one of these enzymes might a better strategy to improve selectivity. More knowledge of specific degradation pathways of individual RGS proteins in specific tissues or under pathophysiologic situations is required for this approach to be successful.

The N-end rule pathway, first proposed by Alexander Varshavsky, is a collection of molecular components that tag proteins bearing an N-degron for proteasomal degradation. This could be considered a "front end" to the ubiquitin/proteasomal degradation pathway. N-degrons are created by proteolytic cleavage of the initial methionine, exposing residue 2 (from a gene numbering perspective) at the N-terminus of a protein. Depending on the nature of this residue it can be stabilizing or destabilizing (Figure 1 and Meinnel et al., 2006; Tasaki and Kwon, 2007; Varshavsky, 1996). N-degrons are, by definition, destabilizing and can be divided into primary, secondary and tertiary destabilizing residues (Figure 1). Destabilizing N-terminal residues are recognized by the N-end rule pathway components, ubiquitylated and targeted to the proteasome. Primary N-degrons are direct targets for degradation, being recognized by E3 ligases. In mammalian cells these are divided into type I (Arg, Lys and His) and type II (Leu, Phe, Trp, Tyr and Ile) denoting specificity for different classes of E3 ligases. Secondary destabilizing residues (Asp, Cys and Glu) first need to be conjugated to Arg by R-transferase (gene name: *ATE-1*) to create primary N-degrons. The third secondary destabilizing residue Cys requires oxidation prior to Arg conjugation. The two tertiary destabilizing residues Asn and Glu are deamidated into the secondary destabilizing residues Asp and Gln, respectively. In mammalian cells, this enzymatic process is carried out by N-terminal amidases specific for either Asn or Glu. The mammalian Asn-specific N-terminal amidase (Ntan1) shows high homology to the *S. cerevisiae* non-specific Nta1p (Grigoryev et al., 1996) but the Glu-specific Nt-amidase (Ntaq1) was only recently cloned and characterized (Wang et al., 2009a).

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

The importance of the N-end rule machinery is demonstrated by the fact that *ATE-1* knock-out mice are embryonic lethal (Kwon et al., 2002). Moreover several types of cancer, including breast cancer, are linked to mutations or altered expression levels of E3 ligases or other components of the ubiquitin/proteasomal pathway (reviewed in (Chen et al., 2006) and (Hoeller et al., 2006).

Several RGS proteins contain a potentially destabilizing N-terminal residue (Fig. 1) and numerous reports support N-end rule-mediated degradation of RGS proteins (Bodenstein et al., 2007; Davydov and Varshavsky, 2000; Lee et al., 2005). Furthermore, RGS protein expression is tightly regulated and is altered in pathophysiological states (Anderson et al., 2007a; Anderson et al., 2007b; Derrien et al., 2003; Song and Jope, 2006; Xie et al., 2009; Zmijewski et al., 2001; Zou et al., 2006).

The best-characterized example of N-end rule pathway-mediated degradation of RGS proteins is RGS4, which has an N-terminal cysteine (a secondary N-degron). The earliest report came from the Varshavsky lab who found RGS4 to be degraded through the N-end rule pathway both *in vitro* and *in vivo* (Davydov and Varshavsky, 2000). Mutants of RGS4 (C2G, C2V and C2A) were stably expressed and not upregulated following treatment with a proteasome inhibitor. They also found RGS16 to be regulated by the N-end rule pathway and subsequent studies have identified RGS5 as another substrate (Bodenstein et al., 2007; Lee et al., 2005). These three proteins all have an N-terminal cysteine followed by a basic residue (lysine or arginine) which, in some systems, is a second target signal for protein degradation. In fact, the RGS4 K3S and RGS8 A2C mutants are stable despite the presence of the N-terminal cysteine (Bodenstein et al., 2007; Davydov and Varshavsky, 2000).

Our lab found differences in the ability of different RGS proteins of the R4 family to inhibit calcium responses elicited by Gq-coupled muscarinic receptor activation in HEK-293 cells (Bodenstein et al., 2007). This difference was largely due to low protein expression of RGS4

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

and RGS5 compared to RGS2 and RGS8. The proteasome inhibitor MG-132 increased protein expression of all RGS proteins studied (RGS2, 4, 5 and 8) with RGS4 showing the greatest increase (>15x) suggesting that these proteins are degraded by the proteasome in HEK-293 cells. Similar to previous studies, a stabilizing mutation in RGS4 (C2S) increased basal protein expression over 50-fold and prevented any further effect of MG-132 treatment, consistent with a role for the N-end rule pathway where cysteine is destabilizing and serine is a stabilizing residue.

Two potentially destabilizing mutations of RGS2, Q2L and Q2R were found in a group of Japanese hypertensive patients (Yang et al., 2005) which is intriguing in light of the role for RGS2 expression in the regulation of blood pressure (Heximer et al., 2003). The mutations are relatively rare (5/1724 hypertensive subjects or 0.3%). We showed in HEK-293 cells that one mutant (RGS2-Q2L) exhibited severely reduced protein expression and markedly increased effects of MG-132 while the other, Q2R, also showed reduced expression but to a lesser extent (Bodenstein et al., 2007). Interestingly, that same study (Yang et al., 2005) found another mutation (R44H, 6/1724 – also 0.3%) that has been shown to reduce membrane localization and function (Gu et al., 2008). Consequently, strategies to pharmacologically enhance RGS2 expression could be quite useful in hypertension and other cardiovascular diseases.

A better understanding of the molecular mechanisms involved in the degradation of specific RGS proteins in particular tissues could provide novel pharmacological targets to enhance their activity. As shown in Fig. 1, there are numerous steps at which this could be accomplished (Ntan1, Ntaq1, ATE1, and various E3 ligases).

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Stabilizing RGS protein expression – implications in pathophysiology

Compelling evidence has come from several recent studies that identified changes in RGS protein expression as a possible pathophysiological mechanism in disease states. Xie et al. identified RGS4 degradation as a possible contributor to breast cancer invasion (Xie et al., 2009). They found that transient expression of wild-type but not a GAP-deficient mutant of RGS4 (N128A) effectively inhibited migration of metastatic breast cancer cells (MDA-MB-231) induced by NIH-3T3 fibroblast conditioned medium. Expression of RGS4 in a xenograft tumor model in nude mice significantly reduced muscle cell invasion with no effect on tumor incidence (Xie et al., 2009). The effect of RGS4 expression was assigned to its regulation of $G\alpha_i$-mediated signaling via the protease-activated receptor 1 (PAR1), which has previously been identified as an important signaling pathway in breast cancer metastasis (Boire et al., 2005). Furthermore, RGS4 protein levels are significantly reduced in human breast carcinomas as demonstrated by immunohistochemical analysis of tissue samples (Xie et al., 2009). Surprisingly, RGS4 mRNA expression is greatly upregulated (>2,000 times) in invasive breast cancer cell lines compared to normal epithelial cells, suggesting that the reduced protein levels of RGS4 is due to markedly increased protein degradation. By inhibiting protein degradation with MG-132, a proteasome inhibitor, RGS4 protein levels were restored and cell migration inhibited (Xie et al., 2009). Together these data indicate that RGS4 protein expression is an important factor in breast cancer metastasis.

RGS2 could also be an interesting target with respect to protein stabilization. As mentioned above RGS2 knock-out mice are hypertensive (Heximer et al., 2003) and human mutations in RGS2 have been identified that are connected to hypertension. The RGS2-Q2L mutation identified in a Japanese population of hypertensive patients (Yang et al., 2005) has reduced stability compared to wild-type in transfected cells (Bodenstein et al., 2007). RGS2 is also a negative regulator of cardiac hypertrophy. α1 Adrenergic receptor stimulation in primary

ventricular myocytes leads to increases in RGS2 mRNA levels while the expression of other RGS proteins (RGS3, 4 and 5) is unaffected. Moreover, overexpression of RGS2 in these cells blocks (Zou et al., 2006) and RNAi-mediated knock-down of RGS2 exacerbates (Zhang et al., 2006) α1 adrenergic-induced hypertrophy. These effects have been assumed to be due to regulation of $G\alpha_q$ signaling. However recent data suggest that there might be additional mechanisms involved.

Chidiac and colleagues (Nguyen et al., 2009) recently demonstrated that RGS2 can regulate protein translation. RGS2 suppressed protein translation *in vitro* by binding to and inhibiting eukaryotic initiation factor 2Bε (eIF2Bε), the rate controlling enzyme in protein translation. Furthermore they found increased levels of protein translation in RGS2 knock-out mice (Nguyen et al., 2009). RGS2 expression, both at the mRNA and protein level, is known to be upregulated by several forms of stress such as oxidative stress, DNA damage, and mechanical stress, situations that are also exhibit reductions in de novo protein synthesis (Song and Jope, 2006; Zmijewski et al., 2001). It is possible that this upregulation of RGS2 could be a way to restore cellular integrity by suppressing protein translation. Furthermore this could be an alternative mechanism for RGS2 to suppress induction of cardiac hypertrophy, which is also characterized by an increase in protein synthesis.

As discussed above, several RGS proteins are involved in regulating the effects of drugs of abuse. An early study identified altered RGS protein expression in response to morphine (Gold et al., 2003). RGS2 and 4 mRNA levels in the LC increased 2-3 fold during opiate withdrawal with a peak at 6h. The levels returned to normal after 24h. As with RGS4 in breast cancer regulation (see above), there were discrepancies between mRNA and protein levels of RGS2 and 4. Protein levels showed a 2-fold increase but had returned to normal levels 6h after opiate withdrawal (Gold et al., 2003). With the potential for both of these proteins to be rapidly degraded via the N-end rule pathway these findings might not be too surprising. The

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

authors of this study argued that increased levels of RGS proteins might be an additional cause of opiate dependence since they can reduce signaling via opiate receptors. The increased RGS protein expression upon withdrawal could therefore explain in part the withdrawal symptoms. The exact molecular mechanism of the upregulation of RGS proteins remains unknown.

Conclusions and future directions

Since their discovery 15 years ago, RGS proteins have emerged as important regulators of GPCR signaling and novel drug targets in a number of pathophysiological states. As more knowledge is obtained about RGS protein function it has become clear that there is more to the story than just accelerating GTPase activity on activated Gα proteins. This review summarizes studies on alterations in RGS protein expression in several diseases and presents an alternative route in drug discovery through regulation of expression and/or allosteric mechanisms. Compounds that specifically stabilize protein expression of certain RGS proteins could potentially be useful in a clinical setting to increase the overall effects of the RGS protein. This would not be limited to the GAP activity, but to all aspects of RGS protein function. Furthermore, given that one RGS protein could be differentially regulated in different cell types, tissues, or pathological states, these approaches might serve to increase specificity. In the case for RGS4, which is expressed in multiple tissues and brain regions, targeting a function specific to one cell type might decrease adverse effects in other tissues. Indeed RGS4 seems to play different roles in different tissues, as described above, with various functions in the brain, cardiovascular system and in the regulation of cancer progression. Much more work is needed to elucidate regulatory mechanisms in specific cellular environments.

References

Adams J (2004) The development of proteasome inhibitors as anticancer drugs. *Cancer Cell* **5**(5):417-421.

Anderson GR, Lujan R, Semenov A, Pravetoni M, Posokhova EN, Song JH, Uversky V, Chen CK, Wickman K and Martemyanov KA (2007a) Expression and localization of RGS9-2/G 5/R7BP complex in vivo is set by dynamic control of its constitutive degradation by cellular cysteine proteases. *J Neurosci* **27**(51):14117-14127.

Anderson GR, Semenov A, Song JH and Martemyanov KA (2007b) The membrane anchor R7BP controls the proteolytic stability of the striatal specific RGS protein, RGS9-2. *J Biol Chem* **282**(7):4772-4781.

Ballon DR, Flanary PL, Gladue DP, Konopka JB, Dohlman HG and Thorner J (2006) DEP-domain-mediated regulation of GPCR signaling responses. *Cell* **126**(6):1079-1093.

Bansal G, Druey KM and Xie Z (2007) R4 RGS proteins: regulation of G-protein signaling and beyond. *Pharmacol Ther* **116**(3):473-495.

Bender K, Nasrollahzadeh P, Timpert M, Liu B, Pott L and Kienitz MC (2008) A role for RGS10 in beta-adrenergic modulation of G-protein-activated K+ (GIRK) channel current in rat atrial myocytes. *J Physiol* **586**(8):2049-2060.

Bernstein LS, Grillo AA, Loranger SS and Linder ME (2000) RGS4 binds to membranes through an amphipathic alpha -helix. *J Biol Chem* **275**(24):18520-18526.

Blazer LL and Neubig RR (2009) Small molecule protein-protein interaction inhibitors as CNS therapeutic agents: current progress and future hurdles. *Neuropsychopharmacology* **34**(1):126-141.

Blazer LL, Roman DL, Chung A, Larsen M, Greedy B, Husbands S and Neubig RR (2010) Reversible, allosteric, small-molecule inhibitors of RGS proteins. *Mol Pharmacol In Press.*

Blundell J, Hoang CV, Potts B, Gold SJ and Powell CM (2008) Motor coordination deficits in mice lacking RGS9. *Brain Res* **1190**:78-85.

Bodenstein J, Sunahara RK and Neubig RR (2007) N-terminal residues control proteasomal degradation of RGS2, RGS4, and RGS5 in human embryonic kidney 293 cells. *Mol Pharmacol* **71**(4):1040-1050.

Boire A, Covic L, Agarwal A, Jacques S, Sherifi S and Kuliopulos A (2005) PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* **120**(3):303-313.

Cao X, Qin J, Xie Y, Khan O, Dowd F, Scofield M, Lin MF and Tu Y (2006) Regulator of G-protein signaling 2 (RGS2) inhibits androgen-independent activation of androgen receptor in prostate cancer cells. *Oncogene* **25**(26):3719-3734.

Cheever ML, Snyder JT, Gershburg S, Siderovski DP, Harden TK and Sondek J (2008) Crystal structure of the multifunctional Gbeta5-RGS9 complex. *Nat Struct Mol Biol* **15**(2):155-162.

Chen C, Seth AK and Aplin AE (2006) Genetic and Expression Aberrations of E3 Ubiquitin Ligases in Human Breast Cancer. *Molecular Cancer Research* **4**(10):695-707.

Chen CK, Eversole-Cire P, Zhang H, Mancino V, Chen YJ, He W, Wensel TG and Simon MI (2003) Instability of GGL domain-containing RGS proteins in mice lacking the G protein beta-subunit Gbeta5. *Proc Natl Acad Sci U S A* **100**(11):6604-6609.

Cho H, Park C, Hwang IY, Han SB, Schimel D, Despres D and Kehrl JH (2008) Rgs5 targeting leads to chronic low blood pressure and a lean body habitus. *Mol Cell Biol* **28**(8):2590-2597.

Cifelli C, Rose RA, Zhang H, Voigtlaender-Bolz J, Bolz SS, Backx PH and Heximer SP (2008) RGS4 regulates parasympathetic signaling and heart rate control in the sinoatrial node. *Circ Res* **103**(5):527-535.

Davydov IV and Varshavsky A (2000) RGS4 Is Arginylated and Degraded by the N-end Rule Pathway in Vitro. *Journal of Biological Chemistry* **275**(30):22931-22941.

Derrien A, Zheng B, Osterhout JL, Ma YC, Milligan G, Farquhar MG and Druey KM (2003) Src-mediated RGS16 tyrosine phosphorylation promotes RGS16 stability. *J Biol Chem* **278**(18):16107-16116.

Drenan RM, Doupnik CA, Jayaraman M, Buchwalter AL, Kaltenbronn KM, Huettner JE, Linder ME and Blumer KJ (2006) R7BP augments the function of RGS7*Gbeta5 complexes by a plasma membrane-targeting mechanism. *J Biol Chem* **281**(38):28222-28231.

Fu Y, Huang X, Piao L, Lopatin AN and Neubig RR (2007) Endogenous RGS proteins modulate SA and AV nodal functions in isolated heart: implications for sick sinus syndrome and AV block. *Am J Physiol Heart Circ Physiol* **292**(5):H2532-2539.

Fu Y, Huang X, Zhong H, Mortensen RM, D'Alecy LG and Neubig RR (2006) Endogenous RGS proteins and Galpha subtypes differentially control muscarinic and adenosine-mediated chronotropic effects. *Circ Res* **98**(5):659-666.

Georgoussi Z, Leontiadis L, Mazarakou G, Merkouris M, Hyde K and Hamm H (2006) Selective interactions between G protein subunits and RGS4 with the C-terminal domains of the mu- and delta-opioid receptors regulate opioid receptor signaling. *Cell Signal* **18**(6):771-782.

Gold SJ, Han MH, Herman AE, Ni YG, Pudiak CM, Aghajanian GK, Liu RJ, Potts BW, Mumby SM and Nestler EJ (2003) Regulation of RGS proteins by chronic morphine in rat locus coeruleus. *Eur J Neurosci* **17**(5):971-980.

Gold SJ, Hoang CV, Potts BW, Porras G, Pioli E, Kim KW, Nadjar A, Qin C, LaHoste GJ, Li Q, Bioulac BH, Waugh JL, Gurevich E, Neve RL and Bezard E (2007) RGS9-2

negatively modulates L-3,4-dihydroxyphenylalanine-induced dyskinesia in experimental Parkinson's disease. *J Neurosci* **27**(52):14338-14348.

Gold SJ, Ni YG, Dohlman HG and Nestler EJ (1997) Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. *J Neurosci* **17**(20):8024-8037.

Grigoryev S, Stewart AE, Kwon YT, Arfin SM, Bradshaw RA, Jenkins NA, Copeland NG and Varshavsky A (1996) A mouse amidase specific for N-terminal asparagine. The gene, the enzyme, and their function in the N-end rule pathway. *J Biol Chem* **271**(45):28521-28532.

Grillet N, Pattyn A, Contet C, Kieffer BL, Goridis C and Brunet JF (2005) Generation and characterization of Rgs4 mutant mice. *Mol Cell Biol* **25**(10):4221-4228.

Gu S, Tirgari S and Heximer SP (2008) The RGS2 gene product from a candidate hypertension allele shows decreased plasma membrane association and inhibition of Gq. *Mol Pharmacol* **73**(4):1037-1043.

Han MH, Renthal W, Ring RH, Rahman Z, Psifogeorgou K, Howland D, Birnbaum S, Young K, Neve R, Nestler EJ and Zachariou V (2009) Brain Region Specific Actions of Regulator of G Protein Signaling 4 Oppose Morphine Reward and Dependence but Promote Analgesia. *Biol Psychiatry*. **67**(8):761-769.

Hepler JR (1999) Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* **20**(9):376-382.

Hershko A and Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67**:425-479.

Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, Oliveira-dos-Santos A, Penninger JM, Muslin AJ, Steinberg TH, Wyss JM, Mecham RP and Blumer KJ (2003) Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest* **111**(4):445-452.

Heximer SP, Watson N, Linder ME, Blumer KJ and Hepler JR (1997) RGS2/G0S8 is a selective inhibitor of Gqalpha function. *Proc Natl Acad Sci U S A* **94**(26):14389-14393.

Hoeller D, Hecker CM and Dikic I (2006) Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat Rev Cancer* **6**(10):776-788.

Hollinger S and Hepler JR (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev* **54**(3):527-559.

Hurst JH and Hooks SB (2009) Regulator of G-protein signaling (RGS) proteins in cancer biology. *Biochem Pharmacol* **78**(10):1289-1297.

Ishii M, Fujita S, Yamada M, Hosaka Y and Kurachi Y (2005) Phosphatidylinositol 3,4,5-trisphosphate and Ca2+/calmodulin competitively bind to the regulators of G-protein-signalling (RGS) domain of RGS4 and reciprocally regulate its action. *Biochem J* **385**(Pt 1):65-73.

Ishii M, Inanobe A, Fujita S, Makino Y, Hosoya Y and Kurachi Y (2001) Ca(2+) elevation evoked by membrane depolarization regulates G protein cycle via RGS proteins in the heart. *Circ Res* **89**(11):1045-1050.

Ishii M, Inanobe A and Kurachi Y (2002) PIP3 inhibition of RGS protein and its reversal by Ca2+/calmodulin mediate voltage-dependent control of the G protein cycle in a cardiac K+ channel. *Proc Natl Acad Sci U S A* **99**(7):4325-4330.

Jayaraman M, Zhou H, Jia L, Cain MD and Blumer KJ (2009) R9AP and R7BP: traffic cops for the RGS7 family in phototransduction and neuronal GPCR signaling. *Trends Pharmacol Sci* **30**(1):17-24.

Jin Y, Zhong H, Omnaas JR, Neubig RR and Mosberg HI (2004) Structure-based design, synthesis, and pharmacologic evaluation of peptide RGS4 inhibitors. *J Pept Res* **63**(2):141-146.

Kaur K, Kehrl JM, Charbeneau RA and Neubig RR (2010) RGS-insensitive Gα subunits: Probes of Gα subtype-selective signaling and physiological functions of RGS proteins. *Methods Mol Biol In Press.*

Kehrl JH, Srikumar D, Harrison K, Wilson GL and Shi CS (2002) Additional 5' exons in the RGS3 locus generate multiple mRNA transcripts, one of which accounts for the origin of human PDZ-RGS3. *Genomics* **79**(6):860-868.

Kimple RJ, De Vries L, Tronchere H, Behe CI, Morris RA, Gist Farquhar M and Siderovski DP (2001) RGS12 and RGS14 GoLoco motifs are G alpha(i) interaction sites with guanine nucleotide dissociation inhibitor Activity. *J Biol Chem* **276**(31):29275-29281.

Kwon YT, Kashina AS, Davydov IV, Hu RG, An JY, Seo JW, Du F and Varshavsky A (2002) An essential role of N-terminal arginylation in cardiovascular development. *Science* **297**(5578):96-99.

Lee MJ, Tasaki T, Moroi K, An JY, Kimura S, Davydov IV and Kwon YT (2005) RGS4 and RGS5 are in vivo substrates of the N-end rule pathway. *Proceedings of the National Academy of Sciences of the United States of America* **102**(42):15030-15035.

Linder ME and Deschenes RJ (2007) Palmitoylation: policing protein stability and traffic. *Nat Rev Mol Cell Biol* **8**(1):74-84.

Meinnel T, Serero A and Giglione C (2006) Impact of the N-terminal amino acid on targeted protein degradation. *Biol Chem* **387**(7):839-851.

Narayanan V, Sandiford SL, Wang Q, Keren-Raifman T, Levay K and Slepak VZ (2007) Intramolecular interaction between the DEP domain of RGS7 and the Gbeta5 subunit. *Biochemistry* **46**(23):6859-6870.

Neubig RR and Siderovski DP (2002) Regulators of G-protein signalling as new central nervous system drug targets. *Nat Rev Drug Discov* **1**(3):187-197.

Nguyen CH, Ming H, Zhao P, Hugendubler L, Gros R, Kimball SR and Chidiac P (2009) Translational control by RGS2. *J Cell Biol* **186**(5):755-765.

Nunn C, Mao H, Chidiac P and Albert PR (2006) RGS17/RGSZ2 and the RZ/A family of regulators of G-protein signaling. *Seminars in Cell & Developmental Biology* **17**(3):390-399.

Popov SG, Krishna UM, Falck JR and Wilkie TM (2000) Ca2+/Calmodulin Reverses Phosphatidylinositol 3,4,5-Trisphosphate-dependent Inhibition of Regulators of G Protein-signaling GTPase-activating Protein Activity. *Journal of Biological Chemistry* **275**(25):18962-18968.

Rahman Z, Schwarz J, Gold SJ, Zachariou V, Wein MN, Choi KH, Kovoor A, Chen CK, DiLeone RJ, Schwarz SC, Selley DE, Sim-Selley LJ, Barrot M, Luedtke RR, Self D, Neve RL, Lester HA, Simon MI and Nestler EJ (2003) RGS9 modulates dopamine signaling in the basal ganglia. *Neuron* **38**(6):941-952.

Roman D, Blazer LL, Monroy CA and Neubig RR (2010) Allosteric Inhibition of the RGS-Gα Protein-Protein Interaction by CCG-4986. *Molecular Pharmacology In Press.*

Roman DL, Ota S and Neubig RR (2009) Polyplexed Flow Cytometry Protein Interaction Assay: A Novel High-Throughput Screening Paradigm for RGS Protein Inhibitors. *J Biomol Screen* **14**(6):610-619.

Roman DL, Talbot JN, Roof RA, Sunahara RK, Traynor JR and Neubig RR (2007) Identification of small-molecule inhibitors of RGS4 using a high-throughput flow cytometry protein interaction assay. *Mol Pharmacol* **71**(1):169-175.

Roof RA, Jin Y, Roman DL, Sunahara RK, Ishii M, Mosberg HI and Neubig RR (2006) Mechanism of action and structural requirements of constrained peptide inhibitors of RGS proteins. *Chem Biol Drug Des* **67**(4):266-274.

Ross EM and Wilkie TM (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* **69**:795-827.

Roy AA, Baragli A, Bernstein LS, Hepler JR, Hebert TE and Chidiac P (2006) RGS2 interacts with Gs and adenylyl cyclase in living cells. *Cell Signal* **18**(3):336-348.

Saitoh O, Masuho I, Terakawa I, Nomoto S, Asano T and Kubo Y (2001) Regulator of G protein signaling 8 (RGS8) requires its NH2 terminus for subcellular localization and acute desensitization of G protein-gated K+ channels. *J Biol Chem* **276**(7):5052-5058.

Sandiford SL and Slepak VZ (2009) The Gbeta5-RGS7 complex selectively inhibits muscarinic M3 receptor signaling via the interaction between the third intracellular loop of the receptor and the DEP domain of RGS7. *Biochemistry* **48**(10):2282-2289.

Sethakorn N, Yau DM and Dulin NO (2010) Non-canonical functions of RGS proteins. *Cell Signal*. **22**(9):1274-81.

Siderovski DP and Willard FS (2005) The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *Int J Biol Sci* **1**(2):51-66.

Sjögren B, Blazer LL and Neubig RR (2010) Regulators of G Protein Signaling Proteins as Targets for Drug Discovery, in *Progress in Molecular Biology and Translational Science* Elsevier Inc. *In Press.*

Song L and Jope RS (2006) Cellular stress increases RGS2 mRNA and decreases RGS4 mRNA levels in SH-SY5Y cells. *Neurosci Lett* **402**(3):205-209.

Talbot JN, Roman DL, Clark MJ, Roof RA, Tesmer JJG, Neubig RR and Traynor JR (2010) Differential modulation of mu-opioid receptor signaling to adenylyl cyclase by regulators of G protein signaling proteins 4 or 8 and 7 in permeabilised C6 cells is Galpha subtype dependent. *Journal of Neurochemistry* **112**(4):1026-1034.

Tasaki T and Kwon YT (2007) The mammalian N-end rule pathway: new insights into its components and physiological roles. *Trends Biochem Sci* **32**(11):520-528.

Tesmer JJ (2010) Structure and Function of Regulator of G Protein Signaling Homology Domains, in *Molecular Biology of RGS proteins* (Fisher R ed) pp 75-113, Elsevier, Amsterdam.

Tesmer JJ, Berman DM, Gilman AG and Sprang SR (1997) Structure of RGS4 bound to AlF4--activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. *Cell* **89**(2):251-261.

Traynor JR and Neubig RR (2005) Regulators of G protein signaling & drugs of abuse. *Mol Interv* **5**(1):30-41.

Varshavsky A (1996) The N-end rule: functions, mysteries, uses. *Proc Natl Acad Sci U S A* **93**(22):12142-12149.

Wang H, Piatkov KI, Brower CS and Varshavsky A (2009a) Glutamine-Specific N-Terminal Amidase, a Component of the N-End Rule Pathway. *Molecular Cell* **34**(6):686-695.

Wang Q, Liu-Chen LY and Traynor JR (2009b) Differential modulation of mu- and delta-opioid receptor agonists by endogenous RGS4 protein in SH-SY5Y cells. *J Biol Chem* **284**(27):18357-18367.

Wang Y, Lee Y, Zhang J and Young KH (2008) Identification of Peptides That Inhibit Regulator of G Protein Signaling 4 Function. *Pharmacology* **82**(2):97-104.

Williams C and Hill SJ (2009) GPCR Signaling: Understanding the Pathway to Successful Drug Discovery, in *G Protein-Coupled Receptors in Drug Discovery* pp 39-50.

Xie Y, Wolff DW, Wei T, Wang B, Deng C, Kirui JK, Jiang H, Qin J, Abel PW and Tu Y (2009) Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4. *Cancer Res* **69**(14):5743-5751.

Yang H, Zonder JA and Dou QP (2009) Clinical development of novel proteasome inhibitors for cancer treatment. *Expert Opinion on Investigational Drugs* **18**(7):957-971.

Yang J, Kamide K, Kokubo Y, Takiuchi S, Tanaka C, Banno M, Miwa Y, Yoshii M, Horio T, Okayama A, Tomoike H, Kawano Y and Miyata T (2005) Genetic variations of regulator of G-protein signaling 2 in hypertensive patients and in the general population. *J Hypertens* **23**(8):1497-1505.

Yau DM, Sethakorn N, Taurin S, Kregel S, Sandbo N, Camoretti-Mercado B, Sperling AI and Dulin NO (2008) Regulation of Smad-Mediated Gene Transcription by RGS3. *Molecular Pharmacology* **73**(5):1356-1361.

Young KH, Wang Y, Bender C, Ajit S, Ramirez F, Gilbert A and Nieuwenhuijsen BW (2004) Yeast-based screening for inhibitors of RGS proteins. *Methods Enzymol* **389**:277-301.

Zachariou V, Georgescu D, Sanchez N, Rahman Z, DiLeone R, Berton O, Neve RL, Sim-Selley LJ, Selley DE, Gold SJ and Nestler EJ (2003) Essential role for RGS9 in opiate action. *Proc Natl Acad Sci U S A* **100**(23):13656-13661.

Zhang W, Anger T, Su J, Hao J, Xu X, Zhu M, Gach A, Cui L, Liao R and Mende U (2006) Selective loss of fine tuning of Gq/11 signaling by RGS2 protein exacerbates cardiomyocyte hypertrophy. *J Biol Chem* **281**(9):5811-5820.

Zhong H and Neubig RR (2001) Regulator of G protein signaling proteins: novel multifunctional drug targets. *J Pharmacol Exp Ther* **297**(3):837-845.

Zmijewski JW, Song L, Harkins L, Cobbs CS and Jope RS (2001) Oxidative stress and heat shock stimulate RGS2 expression in 1321N1 astrocytoma cells. *Arch Biochem Biophys* **392**(2):192-196.

Zou MX, Roy AA, Zhao Q, Kirshenbaum LA, Karmazyn M and Chidiac P (2006) RGS2 is upregulated by and attenuates the hypertrophic effect of alpha1-adrenergic activation in cultured ventricular myocytes. *Cell Signal* **18**(10):1655-1663.

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work is supported by NIH [R01-GM39561] and The Swedish Research council [VR-524-2008-6963].

Molecular Pharmacology Fast Forward. Published on July 27, 2010 as DOI: 10.1124/mol.110.065219
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure 1. Role of the N-end rule pathway in RGS protein degradation. The N-end rule pathway of protein degradation is based on destabilizing residues in the N-terminal of a protein. These are represented with standard one letter amino acid codes. The primary destabilizing residues (green) are ubiquitylated by a series of enzymes initially identified in yeast, N-recognin (Ubr1p, E3), Ub-conjugating enzyme (Ubc2p, E2) and Ub-activating enzyme (Uba1p, E1) and then are targeted for proteasomal degradation. The secondary residues (yellow; Aspartic acid, D and Glutamic acid, E) must first be arginylated by R-transferase (mammalian ATE-1) to create primary N-degrons. The third secondary destabilizing residue, Cysteine (C) is oxidized prior to arginylation. Finally, the tertiary destabilizing residues (red) N and Q are converted to secondary residues by the mammalian N-terminal amidases specific for Asparagine (Ntan1) and Glutamine (Ntaq1), respectively. Several RGS proteins are predicted substrates for this pathway based on their N-terminal residues (indicated in the figure). RGS4, RGS5 and RGS16 (purple) are so far the only RGS proteins confirmed to be degraded by this pathway (Bodenstein et al., 2007; Davydov and Varshavsky, 2000; Lee et al., 2005). Also shown are native RGS proteins predicted to be stable as well as mutant RGS proteins that are either stabilized or have been shown to be degraded through the proteasome (Bodenstein et al., 2007).

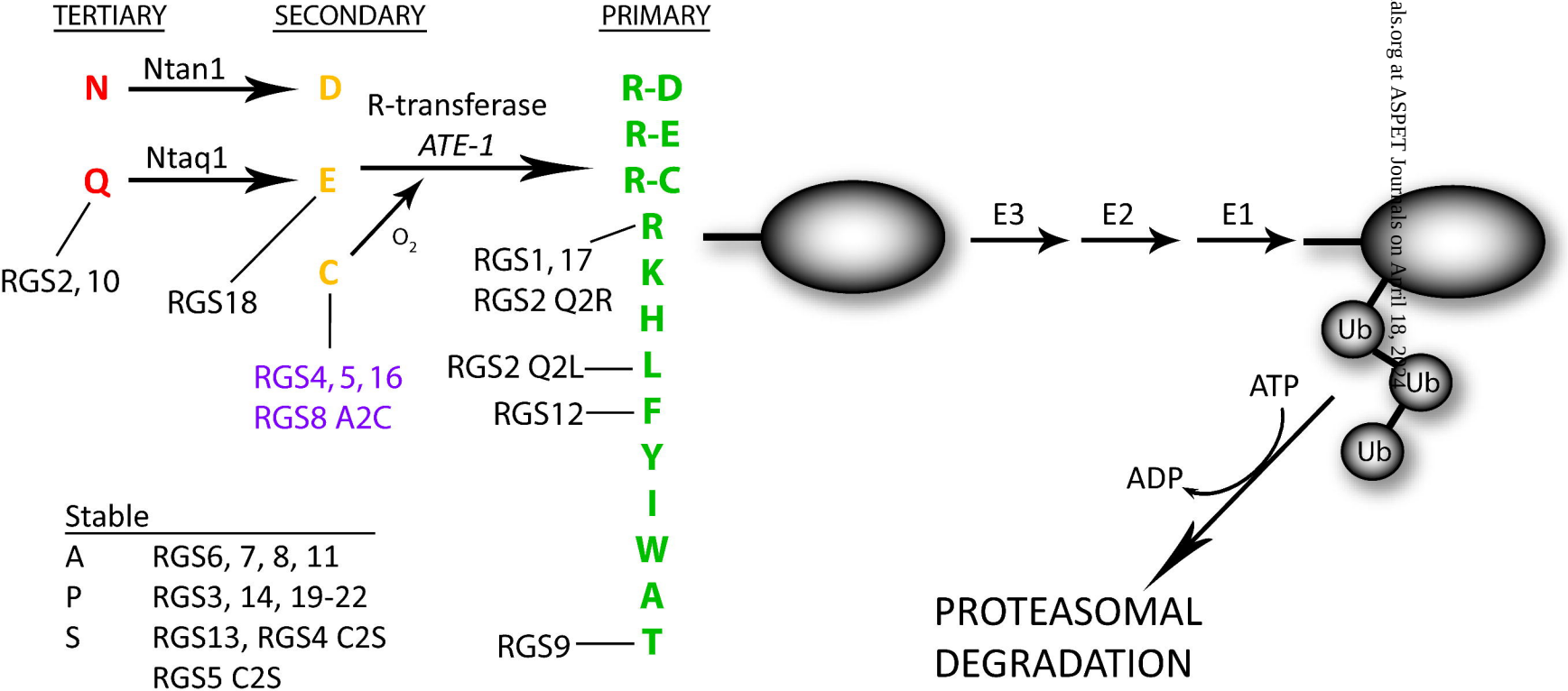

Figure 1