Regulation of the Subcellular Localization of the G-protein Subunit Regulator GPSM3 through Direct Association with 14-3-3 Protein

G-protein signaling modulator-3 (GPSM3), also known as G18 or AGS4, is a member of the Gαi/o-Loco (GoLoco) motif containing proteins. GPSM3 acts through its two GoLoco motifs to exert GDP dissociation inhibitor activity over Gα subunits; recently revealed is the existence of an additional regulatory site within GPSM3 directed toward monomeric Gβ subunits during their biosynthesis. Here, using in silico and proteomic approaches, we have found that GPSM3 also interacts directly with numerous members of the 14-3-3 protein family. This interaction is dependent on GPSM3 phosphorylation, creating a mode II consensus 14-3-3 binding site. 14-3-3 binding to the N-terminal disordered region of GPSM3 confers stabilization from protein degradation. The complex of GPSM3 and 14-3-3 is exclusively cytoplasmic, and both moieties mutually control their exclusion from the nucleus. Phosphorylation of GPSM3 by a proline-directed serine/threonine kinase and the resultant association of 14-3-3 is the first description of post-translational modulation of GPSM3, a hematopoietic-restricted G-protein subunit regulator.

**Background:** GPSM3, a guanine nucleotide dissociation inhibitor acting on Gα, subunit family members, is both cytoplasm- and nucleus-localized.

**Results:** GPSM3 interacts directly with 14-3-3 through a phosphorylation-dependent mechanism.

**Conclusion:** Interaction with 14-3-3 stabilizes a cytoplasmic pool of GPSM3.

**Significance:** Our findings represent the first demonstration of post-translational modulation of GPSM3, a hematopoietic-restricted G-protein subunit regulator.

G-protein-coupled receptors regulate numerous essential biological functions. They are key signal transducers of thousands of extracellular stimuli, regulating a plethora of cellular outcomes (1–4). Typically, during the cycle of activation, the ligand-occupied G-protein-coupled receptor promotes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by the heterotrimeric G-protein α subunit. This active, GTP-bound Gα subunit will thus dissociate from the Gβγ dimer and modulate intracellular effectors such as adenyl cyclases and phospholipases (5, 6). Signal termination is regulated by the action of “regulator of G-protein signaling” (RGS) proteins that enhance the intrinsic GTPase activity of the Gα subunit (7, 8). The inactive, GDP-bound Gα subunit is generally thought to reassociate with the Gβγ dimer to form the inactive heterotrimer. However, a more recently discovered family of G-protein modulators, containing the conserved GoLoco motif, also interacts with inactive, GDP-bound Gα subunits of the Gα subfamily known to inhibit adenyl cyclases (8–10). The GoLoco motif interaction inhibits spontaneous GDP release by the bound Gα subunit (11, 12) and also occludes its Gβγ binding site (13), thereby inhibiting reassociation with Gβγ (14).

In mammalian cells, several different GoLoco motif-containing proteins are expressed, including RGS12 and -14, Rap1GAP, Pcp-2, AGS3, and LGN (8, 15–17). The latter protein is particularly important for spindle orientation during asymmetric cell division (reviewed in Ref. 10). Another protein containing this motif, called GPSM3, has a restricted expression profile to hematopoietic cell lineages (18–20). Recently, GPSM3 was found not only to modulate heterotrimeric G-protein subunit signaling through its two active GoLoco motifs but also to affect monomeric Gβ subunit biosynthesis and stability (20). In the same yeast two-hybrid screen that identified Gβ subunits as GPSM3-interacting proteins (20), we also identified multiple isoforms of 14-3-3 as potential GPSM3 binding partners. 14-3-3 proteins are conserved regulatory molecules expressed in all eukaryotic cells (21, 22). Seven 14-3-3 isoforms

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The abbreviations used are: GoLoco, Gαi/o-Loco; GPSM3, G-protein signaling modulator-3; GSK3, glycogen synthase kinase 3; BRET, bioluminescence resonance energy transfer; RLuc, Renilla luciferase; BiFC, bimolecular fluorescence complementation; Ni-NTA, nickel-nitrilotriacetic acid; pS, phosphoserine; SPR, surface plasmon resonance.
have been identified in mammals (α/β, γ, ε, δ/ζ, η, θ, and σ). 14-3-3 proteins are ubiquitously expressed, although different isoforms show some degree of tissue specificity (21). Multiple proteins have been reported to interact with 14-3-3 isoforms; these 14-3-3 interactors are known to regulate a wide range of cellular activities such as cell signaling, cytoskeleton organization, cellular trafficking, cell proliferation, apoptosis, metabolic pathways, and others (21, 22). Here, we have validated the GPSM3/14-3-3 interaction, mapped the region on GPSM3 critical for this interaction, and examined the relationship between both proteins on their subcellular localization.

**EXPERIMENTAL PROCEDURES**

**Commercial Antibodies, Constructs, and Other Reagents—**
Prodynorphin 109-270 (14-3-3) horseradish peroxidase (HRP)-conjugated anti-hemagglutinin (HA) monoclonal antibody (clone 3F10) was obtained from Roche Applied Science. Anti-β-actin, anti-FLAG M2 antibody, and agarose-conjugated anti-FLAG M2 antibody were purchased from Sigma. HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were from GE Healthcare. Polyclonal antibody against 14-3-3 was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-GPSM3 antibody was produced in-house and has been previously described (20). All antibodies used in this study were cloned in the pcDNA3.1 backbone vector (Invitrogen), with FLAG or HA epitope tag sequences included in the forward PCR primer to produce N-terminal tagged open reading frames as described previously (20) or in the reverse PCR primer to produce C-terminal tagged antibody/bead complexes were then washed with 0.1% Triton X-100 plus PBS for 30 min at room temperature. Nonspecific binding was blocked with 0.1% Triton X-100 plus PBS containing 5% nonfat dry milk for 30 min at room temperature. Cells were then washed with PBS and permeabilized with 0.1% Triton X-100 plus PBS for 10 min at room temperature. Cells were washed twice with PBS, and then resuspended in BRET buffer (phosphate-buffered saline with 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% glucose), and distributed in white 96-well microplates. BRET was initiated by adding coelenterazine-400a at a final concentration of 5 μM. Measurements of emitted light were collected on a Mithras LB-940 plate reader (Berthold Technologies) using a BRET² filter set.

**Bimolecular Fluorescence Complementation (BiFC) —**
Fusion constructs were made similar to those previously described for Gβ/GPSM3 tracking (20): namely, a fusion of the N-terminal fragment (amino acids 1–158) of yellow fluorescent protein (YN) to the N-terminus of full-length GPSM3 (YN-GPSM3) and the C-terminal fragment (amino acids 159–238) of YFP (YC) to the C-terminus of full-length GPSM3 (YC-GPSM3) and cells were incubated at 37 °C for 24 h. Total DNA quantity was normalized using empty pcDNA3.1 vector DNA. To measure fluorescence from formed complexes, transfected cells were washed, harvested, and resuspended in PBS. BiFC signal was acquired using a Mithras LB-940 plate reader using an excitation/emission filter set of 485 and 510 nm. The level of expression of each fusion protein was quantified by Western blotting using a polyclonal antibody directed against the GFP.

**Immunofluorescence Microscopy —**
Cells were seeded in a 12-well plate and transfected with 0.4 μg of each DNA: YN-GPSM3 and 14-3-3-YC. The following day, cells were transfected to a poly-D-lysine-coated coverslip in a 6-well plate and grown overnight. Cells were then fixed with 4% paraformaldehyde plus PBS for 10 min at room temperature. Cells were then washed with PBS and permeabilized with 0.1% Triton X-100 plus PBS for 30 min at room temperature. Nonspecific binding was blocked with 0.1% Triton X-100 plus PBS containing 5% nonfat dry milk for 30 min at room temperature.
Cells were then incubated with an anti-HA FITC conjugate antibody (Roche Applied Science) for 1 h at room temperature in PBS supplemented with 5% nonfat dry milk. Cells were then washed three times with permeabilization buffer. Finally, coverslips were mounted using VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and examined by inverted fluorescence microscopy (Olympus IX70) using a 40× objective.

**Protein Purification and Surface Plasmon Resonance—**Full-length, N-terminal His-tagged, human 14-3-3ζ protein was produced recombinantly in bacteria from a pET vector-based prokaryotic expression construct using previously described cloning, expression, and purification techniques (24). His6-14-3-3 protein was produced recombinantly in bacteria from a pET vector-based cloning, expression, and purification techniques (24). His6-14-3-3 protein was produced recombinantly in bacteria from a pET vector-based cloning, expression, and purification techniques (24).

**Results and Discussion**

**Interaction between GPSM3 and 14-3-3 Family Members Stabilizes GPSM3**—The N-terminal 50 amino acids of GPSM3 are of low complexity and predicted (44) to be disordered (e.g. supplemental Fig. S1). Truncation of the first 30 amino acids has little or no effect on ectopic GPSM3 expression (Fig. 1A). However, N-terminal deletion beyond 30 amino acids was observed to dramatically reduce levels of expressed GPSM3. Analysis of the primary sequence revealed the presence of a potential binding site for 14-3-3 between amino acids 31 and 40 (supplemental Fig. S2A). 14-3-3 binding sites are often localized within disordered regions of a protein; it is thought that interaction with disordered regions facilitates ligand capture and transition from a disordered to ordered state that stabilizes complex formation (25, 26).

As described previously (20), a yeast two-hybrid screen of a human leukocyte cDNA library was performed to identify novel interacting partners of GPSM3; as shown in Fig. 1B, numerous clones and isoforms of 14-3-3 were identified in this screen. Fig. 1C demonstrates the specific interaction between full-length GPSM3 as bait and one of the retrieved 14-3-3 clones, identified as the full-length 14-3-3 isoform. An additional approach taken to identify GPSM3 binding partners, also described previously (20), employed tandem mass spectrometry identification of proteins present in an immunoprecipitated FLAG-GPSM3 and co-immunoprecipitated proteins were resolved by 4–12% SDS-PAGE, proteins visualized by SYPRO Ruby staining, and an apparent band at ~30–40 kDa was excised for LC/MS/MS peptide sequence identification. All 14-3-3 hits and their spectral counts are shown.

![Graph](image.png)

**FIGURE 1. Interaction between GPSM3 and multiple 14-3-3 family members. A, truncation of the GPSM3 N terminus reveals a region between amino acids 31 and 40 essential for stable expression. HEK293 cells were transfected with N-terminal FLAG-tagged GPSM3 expression vectors encoding the indicated amino acid spans. Lysates were resolved by 4–12% SDS-PAGE and immunoblotted with anti-FLAG and anti-β-actin antibodies. B, a yeast two-hybrid screen was performed using full-length GPSM3 as bait on a human leukocyte cDNA library (20). Four different 14-3-3 isoforms, in single or multiple copies, were identified as shown. C, Saccharomyces cerevisiae yeast were co-transformed with the indicated bait plasmids (either expressing the Gal4p DNA binding domain alone (pAS2-1) or as a fusion with full-length GPSM3 (pAS2-1/GPSM3)) and prey plasmids (either expressing the Gal4p activation domain alone (pGAD424) or as a fusion with the entire human 14-3-3 open reading frame (pACT2/14-3-3)). Transformed yeast were plated onto synthetic defined agar (Yc Leu+ Trp+ Ade+ Trp- medium also deficient in adenine (Ade-) and histidine (His-) indicates a positive protein-protein interaction (right panel), D, tandem mass spectrometry analysis confirmed the promiscuous interaction of GPSM3 with multiple isoforms of 14-3-3. Immunoprecipitated FLAG-GPSM3 and co-immunoprecipitated proteins were resolved by 4–12% SDS-PAGE, proteins visualized by SYPRO Ruby staining, and an apparent band at ~30–40 kDa was excised for LC/MS/MS peptide sequence identification. All 14-3-3 hits and their spectral counts are shown.
sus motifs, but numerous 14-3-3 binding partners contain sequences that differ significantly from these optimal motifs (27). On the other hand, site 1 possesses all requirements for an optimal mode II 14-3-3 interaction (supplemental Fig. S1). Moreover, tandem mass spectrometry analyses of post-translational modifications on immunoprecipitated GPSM3 indicated that serines 35, 39, and 153 and threonine 157 are phosphorylated under basal conditions (e.g., supplemental Fig. S3). These two phosphorylated-residue clusters, Ser-35/Ser-39 and Ser-153/Thr-157, represent two of the three potential 14-3-3 interaction sites (namely, sites 1 and 3).

Mapping the Interaction between GPSM3 and 14-3-3 and Its Consequence on GPSM3 Stability—To confirm whether GPSM3 interacts with 14-3-3 proteins and identify the motif involved, we introduced point mutations into full-length, FLAG-tagged GPSM3 and its interaction with HA-tagged 14-3-3 was assessed by co-immunoprecipitation. We first observed that point mutation of arginines 31 and 34 (R31,34A),\textsuperscript{3} serine 35 (S35A), or serine 39 (S39A) within GPSM3 each reduced the level of ectopically expressed GPSM3 protein observed (Fig. 2A); doubling the quantity of cDNA of each mutant resulted in a similar level of expression to that of wild-type GPSM3. We then used these altered transient transfection conditions to test their relative capability to co-immunoprecipitate with HA-tagged 14-3-3. The R31,34A and S35A point mutants of GPSM3 failed to interact with 14-3-3, and a considerable reduction of the interaction was observed with the S39A mutant (Fig. 2B and C); these observations confirm site 1 of GPSM3 as important to the binding of 14-3-3. Mutations to GPSM3 residue serine 35 or 39 to aspartic acid was ineffective in providing a phosphomimetic residue (Fig. 2C). Two additional point mutants of 14-3-3 (R31,34A),\textsuperscript{3} serine 35 (S35A), or serine 39 (S39A) within 14-3-3 were also observed to affect its interactions with the α/β and η isoforms of 14-3-3, whereas the S39A mutation reduced interaction with 14-3-3 α/β and ζ isoforms but had little effect with the 14-3-3 η isoform (Fig. 2C, D, and E). As already described for other 14-3-3 substrates (27), mutation of GPSM3 residue serine 35 or 39 to aspartic acid was found to be ineffective in providing a phosphomimetic residue that enhances 14-3-3 binding (Fig. 2D, C, D, and E). No effect on 14-3-3 binding was observed with S56A, S153A, or T157A mutations to GPSM3 (Fig. 2B), excluding the other two sites identified in silico.

Arginine 56 within 14-3-3ζ is essential for phosphoserine recognition (28–30). We observed that the interaction with wild-type GPSM3 was considerably reduced by introducing a R56A mutation into HA-tagged 14-3-3ζ (Fig. 2F); when combined with the partial loss-of-function S39A GPSM3 mutant, the R56A 14-3-3ζ mutation completely abolished the interaction (Fig. 2F).

Two additional point mutants of 14-3-3ζ isoforms have been well characterized; mutation of serine 58, known to be phosphorylated by PKA or PKB/AKT1, to alanine (S58A) induces constitutive dimerization of 14-3-3ζ, whereas mutation of this serine to aspartic acid (S58E) abrogates dimerization (31–33). However, as shown in Fig. 2G, neither of these mutations to serine 58 affected interaction with GPSM3, suggesting that the GPSM3 interaction with 14-3-3 is independent of the dimerization status of 14-3-3. Homo- and heterodimerization of 14-3-3 proteins is thought to be important for their cellular functions (33). The 14-3-3 dimer adopts a cup-like shape that can interact simultaneously with two binding sites within the same protein, thereby inducing conformational change and regulating protein activity or regulating other interactions via competition or occlusion. The presence of only one 14-3-3 binding site in GPSM3 and the binding of either dimeric or monomeric 14-3-3 to GPSM3 suggest that the 14-3-3 interaction could represent an important scaffolding function and/or stabilize formation of a multiprotein complex (21, 25).

The decreased stability of the 14-3-3 binding loss-of-function GPSM3 mutant R31,34A was further explored. FLAG-tagged GPSM3 was expressed in HEK293 cells in the presence or absence of 200 μg/ml cycloheximide to block de novo protein synthesis, and GPSM3 protein levels were subsequently tracked over time by Western blotting. Only prolonged incubation (>12 h) with cycloheximide was seen to dramatically reduce the protein level of wild-type GPSM3 (Fig. 3). In contrast, the R31,34A mutant of GPSM3 was found to have reduced expression following 6 h of cycloheximide treatment and was almost undetectable at 12 h after treatment (Fig. 3), indicating that the loss-of-function mutant has reduced stability as compared with wild-type GPSM3.

Endogenous GPSM3 Associates with Endogenous 14-3-3 in a Monocytic Cell Line—GPSM3 is proposed to be a hematopoietic restricted protein, and we have previously shown that the human monocytic cell line THP-1 expresses detectable levels of endogenous GPSM3 (20). The interaction between endogenous GPSM3 and 14-3-3 proteins was confirmed in THP-1 cells using co-immunoprecipitation with an in-house anti-GPSM3 monoclonal antibody as well as a polyclonal pan-14-3-3 antibody (Fig. 2H).

Characterization of the Interactions between GPSM3 and Its Binding Partners Using BRET—We also examined the interactions between GPSM3, 14-3-3, and G-protein subunits in a cellular context using BRET between RLuc- and green fluorescent protein (GFP10)-tagged fusions. Saturation BRET has been established as a robust measure of the specificity of an interaction (34). HEK293 cells transfected with a constant amount of 14-3-3-expressing donor construct (14-3-3-RLuc) and increasing amounts of the GPSM3 acceptor fusion expression vector (GFP10-GPSM3) were observed to produce a net BRET signal resulting in a saturable curve (Fig. 4A); mutation of the two arginines within the 14-3-3 binding site of GPSM3 (mutant R31,34A; Fig. 2C) eliminated BRET under the same experimental conditions (Fig. 4A). In addition, S35A and S39A point mutations within this site 1 in GPSM3 also greatly reduced the observed BRET signal as compared with the use of wild-type GPSM3 (Fig. 4B), confirming the results obtained by co-immunoprecipitation (Fig. 2B).

We previously reported that GPSM3 associates with heterotrimeric G-protein β subunits (Gb) during their biosynthetic pathway toward formation of the Gβγ heterodimer (20). This GPSM3/Gβ interaction was found to be independent of the well established Ga/βγ interactions mediated by two of three GoLoco motifs within GPSM3 (19). We employed the BRET assay to evaluate the effect of 14-3-3 on both of these

\textsuperscript{3} R31,34A = R31A and R34A.
G-protein subunit interactions. Overexpression of 14-3-3 had no effect on the BRET ratio generated by co-expression of G\(\alpha_{17}\)-RLuc/GFP10-GPSM3 fusion pairs or G\(\beta_{1}\)-RLuc/GFP10-GPSM3 fusion pairs (Fig. 4, C and D). Conversely, overexpression of G\(\beta_{1}\) was seen to greatly reduce the interaction between GPSM3 and 14-3-3, as shown both by BRET (Fig. 4E) and by co-immunoprecipitation studies (Fig. 4F). The mechanism underlying this unidirectional modulation...
Dynamic Localization of GPSM3 Regulated by 14-3-3

GPSM3 and 14-3-3 Mutually Affect Their Subcellular Localizations—GPSM3 expression has previously been observed throughout the cell including the nucleus, either when overexpressed or at the endogenous level (20); therefore, we next evaluated whether the interaction with 14-3-3 could regulate the subcellular localization of GPSM3. mCherry-tagged, wild-type GPSM3 was seen to be distributed throughout transfected cells, including the nucleus, but with a more pronounced cytoplasmic localization than that seen in the nucleus (Fig. 7A). In contrast, the 14-3-3 binding mutant of GPSM3 (R31,34A; as an mCherry fusion) was observed to be evenly distributed throughout the cytoplasm and completely excluded from the nucleus. This pattern of subcellular localization did not change over time after transfection and was not affected by the level of expression (e.g. Fig. 6B).

Involvement of GSK3α Kinase in the GPSM3/14-3-3 Interaction—Based on in silico prediction and mutagenesis as presented above, GPSM3 interacts with 14-3-3 through a mode II consensus 14-3-3 binding sequence R3–PWRR34S35AP. Mutation of serine 35 completely abrogates the 14-3-3 interaction (e.g. Fig. 2, B–F), suggesting that phosphorylation of serine 35 is obligatory for 14-3-3 binding; the neighboring residue serine 39 also appears important as the interaction is greatly reduced when serine 39 is mutated (Fig. 2).

In silico analyses of potential serine/threonine kinases that could phosphorylate serine 35 (e.g. Scansite) reveal the mode II consensus 14-3-3 binding sequence within GPSM3 (R31PWRR34S35APS39PP) as overlapping with a consensus site for the glycogen synthase kinase 3 (GSK3) substrates (S/T)XXX(pS/T); Ref. 36), where the first S/T is the GSK3 phos-
phorylation target, X is any amino acid, and pS/T is the priming phosphorylation site. GSK3 has an unusual preference for substrates that are prephosphorylated at a priming, C-terminal Ser/Thr residue; this priming process, although not strictly required, increases phosphorylation efficiency of the N-terminal Ser/Thr by 100–1000-fold (37). Moreover, GSK3 favors the presence of proline in the recognition site; for example, one of the GSK3 phosphorylation sites in glycogen synthase is SVPPpS (38). GPSM3 serine 35, required for the 14-3-3 interaction, fits perfectly into this GSK3 recognition sequence. Furthermore, serine 39 was found to be phosphorylated in the mass spectrometry analysis of GPSM3 post-translational modifications (supplemental Fig. S3), and mutation of the serine 39 to alanine (S39A) reduces the GPSM3/14-3-3 interaction. As GPSM3 serine 39 is followed by a proline, we focused our studies on GSK3, a potential proline-directed kinase (39–41), and other well characterized, proline-directed serine/threonine kinases (e.g. cyclin-dependent kinases (CDKs), c-Jun N-terminal kinases (JNKs), p38, and extracellular-signal-regulated kinases 1/2 (ERK1/2)).

FIGURE 4. Characterization of the GPSM3/14-3-3 interaction using BRET. A, the interaction between GPSM3 and 14-3-3 was confirmed by a BRET saturation experiment. A constant amount of 14-3-3-RLuc fusion expression vector was co-transfected into HEK293 cells with increasing amounts of GFP10-GPSM3 fusion expression vector; the resultant net BRET ratio was plotted as a function of the acceptor/donor ratio. The specificity of the interaction is indicated by the saturable nature of the BRET signal. B, point mutations, previously found by co-immunoprecipitation to disrupt (S35A) or strongly reduce (S39A) the GPSM3 interaction with 14-3-3, were introduced in the GFP10-GPSM3 construct and assayed by BRET for 14-3-3 interaction. C and D, the effect of 14-3-3 on the previously described interactions between GPSM3 and Gα1 or Gβ1 (20) was assayed by BRET. Gα1-RLuc or Gβ1-RLuc expression constructs were co-transfected in HEK293 cells with GFP10-GPSM3 (wild type or R31,34A mutant) in the presence or absence of 14-3-3 expression vector. E and F, Gβ1, overexpression reduces interaction between GPSM3 and 14-3-3. E, nontagged Gβ1 was co-expressed with the 14-3-3-RLuc/GFP10-GPSM3 BRET pair, and the effect on BRET signal was measured. Error bars in panels A–E indicate S.E. F, FLAG-tagged GPSM3 (WT or R31,34A) constructs were co-transfected with HA-tagged, wild-type 14-3-3 expression vector in the presence or absence of HA-tagged Gβ1, and immunoprecipitation was performed using agarose-conjugated anti-FLAG M2 antibody. Immunoprecipitates (IP) were resolved on a 4–12% SDS-PAGE and immunoblotted (IB) with anti-HA- and anti-FLAG-HRP conjugates.
As shown in Fig. 8A, neither U0126 (MEK1/2 inhibitor, an upstream activator of ERK1/2) nor SB203580 (p38/H9251/H9252 inhibitor) had any effect on GPSM3 expression levels, even at high concentration (10^7 M). SP600125 (JNK inhibitor), olomoucine (CDK inhibitor), and H89 (a broad range inhibitor often used to inhibit PKA) were each seen to reduce GPSM3 expression levels. Most dramatically, the GSK3/H9251/H9252 inhibitor (SB216763) was found to profoundly reduce the level of ectopic GPSM3 expression (Fig. 8A).

A similar effect was observed on endogenous GPSM3 expression in THP-1 cells, with expression reduced by more than 75% (Fig. 8B). None of the other, above mentioned inhibitors were seen to have any effect on endogenous GPSM3 levels in THP-1 cells, although H89 treatment was found to drastically reduce THP-1 cell viability.

Mammalian cells express two isoforms of GSK3, GSK3α and GSK3β, which share 98% sequence similarity in their kinase domain. However, the isoforms are structurally and functionally distinct (42). There is an N-terminal glycine-rich extension in
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GSK3α as well as distinct C-terminal regions (only 38% similarity between isoforms). When co-expressed in HEK293 cells, GSK3α, but not GSK3β, was found in complex with FLAG-tagged GPSM3 (Fig. 8C). The exclusion of GSK3β, even when overexpressed, highlights the unique nature and specificity of this GSK3α/GPSM3 interaction. To date, only the protein adaptor RACK1 has been shown to selectively interact with GSK3α, an interaction that involves the unique N-terminal extension of GSK3α (43). The mechanism by which GSK3α is recruited to GPSM3 as well as the regulation of this interaction is unknown.

Conclusions—A single 14-3-3 binding site exists in GPSM3 and corresponds to a consensus mode II binding motif, RX(ϕ/S)(+)(p5)XP, in which position P0 is an obligatory ϕ5 known to interact with positively charged arginine 56 and 127 within the 14-3-3 binding groove (4 isofrom numbering). The arginine at position P−4 forms an intramolecular salt bridge with the P0 phosphoserine, and the proline at position P0 orients the main chain direction of the binding peptide (25). The binding site within GPSM3 (RPWRS35AP) contains all requirements for an optimal, high affinity interaction; mutagenesis confirmed the critical role of these elements as the R31,34A and S35A mutants of GPSM3 completely abolish interaction with 14-3-3. Additionally, the R56A 14-3-3 mutant exhibited reduced interaction with GPSM3. Co-immunoprecipitation of endogenous GPSM3 and 14-3-3 proteins from the human monocytic cell line THP-1 suggests basal phosphorylation of GPSM3 at serine 35 as potentially mediated by GSK3α. The GPSM3/14-3-3 interaction is seen to stabilize GPSM3 from degradation and also support the nuclear exclusion of both proteins. Future studies will be directed at establishing the specific roles of these newly described 14-3-3 and GSK3α interactions in the G-protein modulatory functions of GPSM3 within immune system cells.

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