The ubiquitin-proteasome system (UPS) plays a critical role in protein degradation. The 19S regulatory particle (RP) of the 26S proteasome mediates the recognition, deubiquitylation, unfolding, and channeling of ubiquitylated substrates to the 20S proteasome. Several subunits of the 19S RP interact with a growing number of factors. The cyclophilin-like domain (CLD) of Ran-binding protein-2 (RanBP2/Nup358) associates specifically with at least one subunit, S1, of the base subcomplex of the 19S RP, but the functional implications of this interaction on the UPS activity are elusive. This study shows the CLD of RanBP2 promotes selectively the accumulation of a subset of reporter substrates of the UPS, such as the ubiquitin (Ub)-fusion yellow fluorescent protein (YFP) degradation substrate, Ub<sub>G76V</sub>-YFP, and the N-end rule substrate, Ub-R-YFP. Conversely, the degradation of endoplasmic reticulum and misfolded proteins, and of those linked to UPS-independent degradation, is not affected by CLD. The selective effect of CLD on the UPS in vivo is independent of, and synergistic with, proteasome inhibitors, and CLD does not affect the intrinsic proteolytic activity of the 20S proteasome. The inhibitory activity of CLD on the UPS resides in a purported SUMO binding motif. We also found two RanBP2 substrates, RanGTPase-activating protein and retinitis pigmentosa GTPase regulator interacting protein-1ε1, whose steady-state levels are selectively modulated by CLD. Hence, the CLD of RanBP2 acts as a novel auxiliary modulator of the UPS activity; it may contribute to the molecular and subcellular compartmentation of the turnover of properly folded proteins and modulation of the expressivity of several neurological diseases.

Surveillance of synthesis and turnover of proteins, and degradation of misfolded and damaged proteins, are fundamental biological processes, which have been linked to the pathogenesis of numerous disorders (1–6). The ubiquitin-proteasome system (UPS) pathway is the major pathway of proteolysis of intracellular proteins (1–6). Multiple lines of evidence support that the 19S regulatory particle (RP; designated also as the 19S cap) of the 26S proteasome is subjected to multiple levels of regulation and likely, remodeling, by loosely associated factors. For example, loss-of-function of the yeast subunit, Rpn10, has modest phenotypic effects (7, 8) and in contrast to its mammalian orthologue, the S5a subunit, Rpn10 lacks one of the two ubiquitin-interacting motifs present in S5a that is required for binding polyubiquitin (9–12). Likewise, the yeast subunits Rpn1, Rpn9, and Rpn2 are not lethal in yeast, the phenotype varies with the genetic background, and they are synthetic lethal (13–16). More importantly, a growing number of factors identified to interact with the 19S RP appear to be critical for substrate targeting and/or docking to the 26S proteasome (2, 12, 17). These auxiliary factors likely define complementary pathways of the UPS and confer a critical layer of specificity to compartmentalize the proteolytic activity of the 26S proteasome. Overexpression of some of these factors inhibits UPS-mediated proteolysis (18, 19). On the other hand, a number of other factors such as Parkin, an E3-ligase, and ataxin-7, interact with specific subunits of the proteasome, such as S5a (Rpn10) and S4 (Rpt2), respectively (20, 21). Human mutations in these accessory proteins are directly implicated in neurodegenerative diseases (e.g. Parkinson disease and spinocerebellar ataxia linked to macular dystrophy) affecting clinically and selectively regions of brain and retina (22–26). Likewise, the deregulation of the UPS is thought to underlie the molecular pathogenesis of several neurological disorders such as prion diseases (27), Huntington (28) and sporadic Parkinson diseases (29), syndromic mental retardation (30–32), age-related macular degeneration (33), and manifestations linked to the aging of the retina (34).

The Ran-binding protein-2 (RanBP2/Nup358) is comprised of multiple structural domains (35–39). Each domain of RanBP2 was found to associate specifically with a number of different partners and these may contribute to the pleiotropic function of RanBP2 (35, 36, 38, 40–49). The RanBP2 partners are implicated in multiple biological processes such as glucose metabolism, surveillance of synthesis and turnover of proteins, and degradation of misfolded and damaged proteins, which have been linked to the pathogenesis of numerous disorders (1–6). The ubiquitin-proteasome pathway is the major pathway of proteolysis of intracellular proteins (1–6). Multiple lines of evidence support that the 19S regulatory particle (RP; designated also as the 19S cap) of the 26S proteasome is subjected to multiple levels of regulation and likely, remodeling, by loosely associated factors. For example, loss-of-function of the yeast subunit, Rpn10, has modest phenotypic effects (7, 8) and in contrast to its mammalian orthologue, the S5a subunit, Rpn10 lacks one of the two ubiquitin-interacting motifs present in S5a that is required for binding polyubiquitin (9–12). Likewise, the yeast subunits Rpn1, Rpn9, and Rpn2 are not lethal in yeast, the phenotype varies with the genetic background, and they are synthetic lethal (13–16). More importantly, a growing number of factors identified to interact with the 19S RP appear to be critical for substrate targeting and/or docking to the 26S proteasome (2, 12, 17). These auxiliary factors likely define complementary pathways of the UPS and confer a critical layer of specificity to compartmentalize the proteolytic activity of the 26S proteasome. Overexpression of some of these factors inhibits UPS-mediated proteolysis (18, 19). On the other hand, a number of other factors such as Parkin, an E3-ligase, and ataxin-7, interact with specific subunits of the proteasome, such as S5a (Rpn10) and S4 (Rpt2), respectively (20, 21). Human mutations in these accessory proteins are directly implicated in neurodegenerative diseases (e.g. Parkinson disease and spinocerebellar ataxia linked to macular dystrophy) affecting clinically and selectively regions of brain and retina (22–26). Likewise, the deregulation of the UPS is thought to underlie the molecular pathogenesis of several neurological disorders such as prion diseases (27), Huntington (28) and sporadic Parkinson diseases (29), syndromic mental retardation (30–32), age-related macular degeneration (33), and manifestations linked to the aging of the retina (34).

The Ran-binding protein-2 (RanBP2/Nup358) is comprised of multiple structural domains (35–39). Each domain of RanBP2 was found to associate specifically with a number of different partners and these may contribute to the pleiotropic function of RanBP2 (35, 36, 38, 40–49). The RanBP2 partners are implicated in multiple biological processes such as glucose metabolism.
catabolism (40), protein biogenesis (35, 43), mitochondria trafficking and function (50), modulation of protein–protein interaction by sumoylation (51–54), and nucleocytoplasmic trafficking (36). We previously identified a novel domain, the cyclophilin-like domain (CLD), in RanBP2, with low yet significant homology to its C-terminal cyclophilin domain (35). We have shown that the CLD of RanBP2 associates specifically with the S1 (p112) subunit of the 19S cap of proteasome, suggesting that RanBP2, via its CLD domain, may modulate the activity of the UPS and protein biogenesis (45). However, the functional implication(s) of the CLD of RanBP2 on the UPS activity remains elusive. In this report, we employ various sensors of the UPS to probe the impact in vivo of the CLD of RanBP2 on the activity of UPS. We found that the CLD strongly promotes the selective accumulation of a subset of UPS sensors and the biogenesis of selective substrates.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The bovine sequence of CLD domain and its variants were amplified by PCR from bovine RanBP2 cDNA using *Pfu* and subcloned into the vector, pDEST733, in-frame with N-terminal tag, red fluorescent protein (RFP), using the Gateway Cloning System (Invitrogen). Point mutations and deletion constructs were generated by PCR from cDNA of RanBP2 with primers harboring the cognate mutated nucleotides and complementary to various regions of CLD. All constructs produced were confirmed by DNA sequencing. RFP sequence in pDEST733 was replaced with that of YFP to generate the vector, pDEST733Y. Bovine retinitis pigmentosa GTPase regulator interacting protein-1 (bRPGRIP1, PA1–969; S10B, PA1–962) from Affinity BioReagents and used as substrates. The ubiquitin-proteasome system (UPS) reporter constructs, pUbG76V-YFP, pUb-R-YFP, were kindly provided by N. Danuser. Polyclonal HK1 was a gift from Steve Wilson; the anti-importin-β (Mab3E9, 0.4 μg/ml) antibody was a gift from Steve Adam, H2 monoclonal antibody (0.4 μg/ml) against bovine brain kinesin heavy chain was a gift from George Bloom; anti-Ubc9 polyclonal antibody (1:500) was a gift from Mary Dasso; NPHP4 (1:1000) was a gift from R. Bloom; monoclonal mitochondrial Hsp70 (MA3-028, 1:1000) and rabbit polyclonal antibodies against proteasome subunits (S1, PA1–973; S3, PA1–974; S4, PA1–965; S5A, PA1–966; S7, PA1–969; S10B, PA1–962) from Affinity BioReagents and used at 0.4 μg/ml; Living Color DsRed polyclonal antibody (1:2000) was purchased from Clontech; antibody against PA700 was a gift from G. DeMartino; monoclonal antibody (0.4 μg/ml) against bovine brain kinesin heavy chain was a gift from Steve Wilson; the anti-importin-β (Mab3E9, 0.4 μg/ml) antibody was a gift from Steve Adam, the mouse antibody against NPHP4 (1:1000) was a gift from R. Hipp, Ab38 against RPGRIP1 (125 ng/ml) was described earlier (58), and the anti-GMP1 (SUMO-1; 400 ng/ml) monoclonal antibody was a gift from R. Bloom.

**Cell Culture and Transfection**—RGC-5 and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a humidified incubator containing 5% CO₂ at 37 °C. Cells were plated in 6-well plates 1 day before transfection using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Cells were either detached by trypsinization for whole cell fluorescence measurement, or lysed directly in SDS-sample buffer for Western blot analysis.

**UPS Reporter Assays**—COS7 cells in 6-well plates co-expressing a reporter construct and a test construct were harvested and washed with phosphate-buffered saline. Cells were resuspended in 330 μl of phosphate-buffered saline per well and three aliquots of 100 μl each were transferred to 96-well plates for fluorescence reading using SpectraMax M5 microplate reader (Molecular Devices). All data shown represent the average of three experiments. Two-tailed equal variance t test was performed. For yellow fluorescence (YFP), excitation/emission/cutoff = 510/530/530 nm; for red fluorescence (RFP), excitation/emission/cutoff = 575/607/590 nm; for green fluorescence (EGFP), excitation/emission/cutoff = 480/510/510 nm.

**20S Proteasome Activity Assay**—To measure the proteasome activity of living cells, COS7 cells were cultured in 6-well plates and transfected with RFP-CLD or empty RFP vector alone. Twenty-four hours later, 100 μM fluorogenic peptide substrate, Suc-LLVY-amc, with or without MG132 (60 μM) or lactacystin (20 μM), was added to the media and incubated for an additional 2 h. Two hundred μl of medium was taken for measurement of free amc (excitation/emission/cutoff = 380/460/455 nm). To measure proteasome activities in cell extracts, cellular protein extraction and proteasome activity measurement were performed as described by Kisselev and Goldberg (56). Proteasome substrates were purchased from BioMol.

**GST Pulldown Assay**—Pulldown assays were described (45, 57). Briefly, cultured RGC-5 and COS7 cells were lysed in CHAPS lysis buffer (20 mM Tris-HCl, pH 6.8, 0.25 M NaCl, 0.75% CHAPS, 5% glycerol, 2 mM β-mercaptoethanol) and centrifuged at 10,000 × g for 20 min at 4 °C. Supernatant was pre-cleared with the incubation of 100 μg/ml GST and glutathione S-agarose beads, and then used for GST pulldown.

**Western Blot Analysis and Antibodies**—Proteins were resolved in SDS-PAGE gel and transferred to Immobilon-P membrane (Millipore). Membrane was blocked by milk and incubated with respective primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody and detection by SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce). The following primary antibodies were used: monoclonal mitochondrial Hsp70 (MA3-028, 1:1000) and rabbit polyclonal antibodies against proteasome subunits (S1, PA1–973; S3, PA1–974; S4, PA1–965; S5A, PA1–966; S7, PA1–969; S10B, PA1–962) from Affinity BioReagents and used at 0.4 μg/ml; Living Color DsRed polyclonal antibody (1:2000) was purchased from Clontech; antibody against PA700 was a gift from G. DeMartino; H2 monoclonal antibody (0.4 μg/ml) against bovine brain kinesin heavy chain was a gift from George Bloom; anti-Ubc9 polyclonal antibody (1:500) was a gift from Mary Dasso; Nup62 was from BD Transduction Laboratories (1:5000); ubiquitin monoclonal antibody (P4D1, 1: 1000) was purchased from Santa Cruz Biotechnology; monoclonal nuclear pore complex protein/RanBP2 antibody clone Mab414 (0.4 μg/ml) was purchased from Covance Babco; monoclonal anti-RanGAP1 (1:1000) was a gift from Elias Coutavas; rabbit polyclonal antibody against NPHP4 (1:1000) was a gift from Steve Wilson; the anti-importin-β (Mab3E9, 0.4 μg/ml) antibody was a gift from Steve Adam, the mouse antibody against NPHP4 (1:1000) was a gift from R. Hipp, Ab38 against RPGRIP1 (125 ng/ml) was described earlier (58), and the anti-GMP1 (SUMO-1; 400 ng/ml) monoclonal antibody was from Invitrogen.

**RESULTS**

The CLD of RanBP2 Selectively Promotes the Accumulation of a Subset of Ubiquitin Fusion Degradation Substrates in Live Cells—We took advantage of a panel of UPS sensors to report the CLD-mediated modulation of various facets of UPS activity.
To evaluate the effect of CLD on the ubiquitin-dependent proteolysis of substrates in living cells, we measured in vivo the steady state levels of four UPS sensors in the presence and absence of CLD. The UPS sensors comprise the YFP reporter fused to various degradation signals of the UPS. The four UPS sensors employed are Ub-R-YFP, UbG76V-YFP, CD3-H9254-YFP, and CL1-YFP. The Ub-R-YFP and UbG76V-YFP contain a ubiquitin moiety fused to YFP. The former is an N-end rule substrate, whose polyubiquitylation and degradation depends on the presence of a free N-terminal arginine residue, whereas the mutation G76V of ubiquitin in UbG76V-YFP prevents its deubiquitylation and uses the ubiquitin moiety as a template for polyubiquitylation (59, 60). The UbG76V-YFP and Ub-R-YFP constructs reflect the degradation of properly folded substrates. The CD3-H9254-YFP measures the endoplasmic reticulum-associated degradation pathway, because the T-cell receptor subunit is an endoplasmic reticulum-associated degradation substrate (60). The CL1-YFP comprises the fusion of YFP to a short degron with a bulky hydrophobic motif resembling a misfolded domain (60, 61). The CL1-YFP generates an unstable YFP and reports the degradation of misfolded proteins by the UPS (60, 61). The UbG76V-YFP and Ub-R-YFP constructs reflect the degradation of properly folded substrates. The CD3-H9254-YFP measures the endoplasmic reticulum-associated degradation pathway, because the T-cell receptor subunit is an endoplasmic reticulum-associated degradation substrate (60). The CL1-YFP comprises the fusion of YFP to a short degron with a bulky hydrophobic motif resembling a misfolded domain (60, 61). The CL1-YFP generates an unstable YFP and reports the degradation of misfolded proteins by the UPS (60, 61). Finally, we also generated a sensor of UPS-independent proteolytic activity, GFP-DE, where GFP is fused to residues 422 to 461 of the degradation and PEST-containing domain of the mouse ornithine decarboxylase (62). Mouse ornithine decarboxylase is degraded in a UPS-independent fashion (62).

As shown in Fig. 1, expression of CLD fused to the monomeric red fluorescent protein (RFP-CLD), but not RFP alone, had a strong and selective impact on the in vivo accumulation of Ub-R-YFP and UbG76V-YFP, but not on any other sensors tested. The highest effect was observed with UbG76V-YFP.

These data support that the CLD selectively suppresses the activity of the UPS on properly folded substrates, because accumulation of all other substrate sensors failed to respond to CLD.
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Functional Analysis of the Flanking Domains, Ran-binding Domain-3 (RBD3) and Internal Repeat-1 (IR1), of CLD of RanBP2, in the Modulation of the UPS Activity—The CLD of RanBP2 is flanked by the upstream RBD3 and the downstream IR1 domains (Fig. 2A). The former is known to associate with Ran-GTP, whereas the latter interacts with the E2-ligase, ubiquitin-conjugating enzyme-9 (ubc9) (46, 47). We confirmed these results with pulldown assays using GST-RBD3-CLD constructs with or without the flanking domain, IR1+2, and CHAPS-solubilized retinal extracts. As shown in Fig. 2B, deletion of IR1+2 did not affect the GTP-dependent association of RanGTPase (lower panel), but it completely abrogated the binding activity of ubc9 to RBD3-CLD domains independently of the absence and presence of nonhydrolyzable nucleotide analogs (upper panel). To evaluate the effect of RBD3 and IR1+2 domains on the activity of CLD toward the UPS in living cells, we followed the steady-state levels of the UPS sensor, UbG76V-YFP, in the presence of CLD constructs with or without its flanking domains (Fig. 2A). As shown in Fig. 2C, all constructs promoted the accumulation of UbG76V-YFP. However, the CLD construct alone exhibited the highest inhibition of the UPS, whereas the removal of the ubc9-interacting domain, IR1+2, from the RBD3-CLD-IR1+2 construct (RFP-RC) did not significantly affect its inhibitory activity on the UPS. On the other hand, the deletion of RBD3 from the RBD3-CLD-IR1+2 construct (RFP-CI) significantly increased the inhibitory activity on the UPS. Collectively, the data support that the inhibitory property to suppress the UPS activity resides with CLD, and the RBD3 plays a significant role in down-regulating the inhibition of the UPS by CLD.

Impact of CLD and 20S Proteasome Inhibitors, MG132 and Lactacystin, on the UPS Activity and Proteolysis of Fluorogenic Peptide Substrates of the 20S Proteasome—In light of the inhibitory effect of CLD on the UPS activity, we carried out a pharmacological approach to discern further between the effect of CLD in vivo on the 20S proteasome and selective UPS activities. We transfected cells with and without CLD of RanBP2, and in the presence and absence of the 20S proteasome inhibitors, MG132 (Fig. 3A) and lactacystin (Fig. 3B) (56). The effect of RFP-CLD on the UPS activity of living cells was normalized to that obtained with RFP alone. The magnitude of the effect of CLD alone on the UPS activity was comparable with that of the proteasome inhibitors alone (~2.5-fold inhibition) (Fig. 3, A and B). However, when cells were concomitantly subjected to transfection of CLD, and proteasome inhibitors, a synergistic inhibitory impact of ~1.5–2-fold on the UPS activity was observed, when compared with either treatments alone (Fig. 3, A and B). These results were also confirmed by Western blot analysis with an anti-ubiquitin antibody of homogenates of transfected cells in the presence and absence of RFP-CLD and the proteasome inhibitor, MG132 (Fig. 3C). We observed that MG132 and RFP-CLD alone caused an increase of UbG76V-YFP (and poly-UbG76V-YFP). However, the RFP-CLD alone did not promote an overall accumulation of poly-Ub substrates. This was in contrast with MG-132, which caused a significant accumulation of a broad mass spectrum of poly-Ub substrates. When combined together, CLD and MG-132 had a synergistic effect on the accumulation of UbG76V-YFP. This outcome lends support that the CLD of RanBP2 has an independent mechanism of action on the UPS that of the 20S proteasome inhibitors.

We carried out experiments also with fluorogenic peptidyl substrates in vitro and in vivo, to corroborate the negative and positive effects of CLD of RanBP2 and proteasome inhibitors, respectively, on the 20S proteasome proteolytic activity. We measured the caspase, chymotryptic, and trypsin proteolytic activities of the 20S proteasome with cell extracts by employing specific fluorogenic peptidyl substrates reporting those specific proteolytic activities (Fig. 4, A–C). All readouts were normalized to that obtained with RFP alone. In contrast to the known specific inhibition of proteolytic activity of each proteasome inhibitor, the presence of CLD did not have an effect on any proteolytic activity of the 20S proteasome. This observation was also confirmed in live cells with a membrane permeable fluorogenic peptide substrate (Suc-LLVY-amc) (56), in the presence and absence of CLD and proteasome inhibitors (Fig. 4D). Again, whereas the proteasome inhibitors had an impact...
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FIGURE 4. Impact of CLD and 20S proteasome inhibitors, MG132 (A) and lactacystin (B), on the proteolysis of fluorogenic peptides measuring specifically caspase-α, chymotrypsin-β, and trypsin-like activities (C), in cell extracts (A–C), and in live cells (D). Conversely to the proteasome inhibitors, the CLD has no impact on any of the specific proteolytic activities of the 20S proteasome.

on the proteolysis of the peptidyl substrate, Suc-LLVY-amc, CLD had no effect in vivo on the 20S proteasome activity. Altogether, these data support further that CLD modulates the activity of the UPS, but not of the 20S proteasome.

Structure-function Analysis of CLD of RanBP2 on the UPS Activity—Because the inhibitory activity on the UPS resides solely with the CLD domain of RanBP2 (Fig. 2C), we carried out structure-function analysis of CLD to identify the minimal domain in CLD comprising its UPS inhibitory activity. Another interesting feature of CLD is that it contains the small ubiquitin-like modifier-1 (SUMO-1)-binding motif (SBM), Val/Ile-Val/Ile-Val/Ile (amino acids 2632–2635), which is reported to associate in vitro and in the presence of ubc9, with the RanGTPase-activating protein covalently modified with SUMO-1 (RanGAP-SUMO-1) produced from reticulocyte extracts (63). Because deletion of the ubc9-interacting domain (I) of RanBP2 from the RBD3-CLD-IR1 + 2 (RCI) construct had no impact on its activity (Fig. 2C), it is important to re-evaluate also the putative role of the SBM of CLD under the context of the UPS activity modulated by CLD. To this effect, we generated a series of N- and C-terminal deletions constructs of CLD (Fig. 5A). Deletion analysis of N- and C-terminal segments of CLD supported that the sequence between residues 2627 and 2646 comprised most of the UPS inhibitory activity of CLD (Fig. 5B). Deletion of residues upstream and downstream of residues 2627 and 2646, respectively, had little or no impact on the accumulation of UbG76V-YFP (Fig. 5, A and B). On the other hand, deletion of residues between amino acids 2627 and 2646 causes a strong suppression of accumulation of UbG76V-YFP (Fig. 5, A and B). Remarkably and as previously noted, the purported SBM, Val/Ile-Val/Ile-Val/Ile-Val/Ile (63), is found between residues 2627 and 2646. Hence, we examined whether mutations in this motif abolish the inhibitory activity of CLD on the UPS. To this effect, we mutated the VLIV motif to VLKA in constructs comprising the whole CLD domain and a small C-terminal segment of 53 residues that retain most of the UPS activity (Fig. 5, A and B). Indeed, we observed the mutations in both constructs were sufficient to suppress completely in vivo the accumulation of UbG76V-YFP and hence, the inhibitory activity of CLD over the UPS. Hence, the structure-function analysis of CLD defines a novel motif in CLD, comprising the purported SBM, with a role in the modulation of the UPS activity.

The SBM of CLD Modulates the Binding Activity of a Subcomplex of the 19S Cap to CLD and CLD Selectively Associates with Ubiquitylated Substrate(s)—To probe further the role of CLD of RanBP2 on the UPS, we examined the association of multiple subunits of the 19S cap of the proteasome with CLD and the impact of the mutation in the SBM on the binding activity toward such subunits. Immunoblots of GST-CLD and -CLDMut coprecipitates of extracts of the retinal ganglion cell line, RGC-5, with antibodies against various subunits of the 19S cap (Fig. 6A). These include the S1, S3, S5a, S7, and S10b, but not S4. In addition, CLD causes a decrease of the electrophoretic mobility of S1 and S3 subunits, and to a lesser extent the S7 and S10a, whereas the migration of S5a was not affected. The association of the S1 and S3 subunits was markedly increased with the CLDMut and this mutant construct specifically promoted an increase of the apparent molecular mass (~2–3 kDa) of the S5a subunit. The increase of binding activity of the S1 subunit to CLDMut and the electrophoretic...
mobility shift of S1, was also confirmed independently with another antibody in two cell lines, RGC-5 and COS7 (Fig. 6B, upper and lower panels). The S5a subunit of the proteasome has been implicated as one of the receptors for ubiquitylated substrates (9, 11, 64, 65). Hence, we evaluated whether the CLD associates with ubiquitylated substrates, because the 19S subcomplex bound to CLD comprises the S5a subunit. Western blot of coprecipitates of cell extracts incubated with GST-CLD and -CLDMut showed that a very restricted number of ubiquitylated protein species of high molecular mass and this species associates stronger with CLDMut (Fig. 6C, upper panel). RanGAP did not coprecipitate with any of the constructs (Fig. 6C, lower panel). Finally, no SUMO-1-conjugated protein species co-precipitated with GST-CLD and -CLDMut constructs in COS7, RGC-5, and retinal extracts (Fig. 6D, and data not shown).

Identification of Two Protein Substrates, RanGAP and RPGRIP1α1, Whose Steady-state Levels Are Modulated by CLD of RanBP2—The finding that the CLD of RanBP2 has a significant and selective effect on the activity of the UPS supports the hypothesis that the CLD modulates the steady-state levels of a selective number of properly folded protein substrates. The identification of such substrates is helpful to discern the role of RanBP2 and its partners in cell function and allied pathological processes. We took a candidate protein approach to seek whether the steady-state levels of RanBP2, many of its partners, and some partners associated with these, were affected by the expression of CLD. Comparison of homogenates of transfected COS7 cells with RFP alone and RFP-CLD did not exhibit any significant differences of most proteins reported to associate directly and indirectly with RanBP2 (Fig. 7A). However, we found two substrates, RanGTPase-activating protein (Ran-GAP) (Fig. 7B) and the retinitis pigmentosa GTPase regulator interacting protein-1α1 isoform (RPGRIP1α1) (Fig. 7C) (55, 66), whose levels where affected strongly by CLD expression. CLD-transfected cells presented selective up-regulation of the endogenous levels of RanGAP, but not of a higher molecular mass isoform of RanGAP that is possibly sumoylated (Fig. 7B). The mutation in the SBM of CLD abolishes the CLD-mediated increase of the steady-state levels of RPGRIP1α1 in vivo. Note that the point mutation in the SBM of CLD increases the steady-state levels of CLDMut comparatively to CLD (compare lanes 2 and 3; inset immunoblot). x axis of C and D represents the amount of transfected DNA.

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levels of RPRGIP1α1 in a concentration-dependent manner. The mutation in the CLD construct abolished the ability of CLD to promote an increase of RPRGIP1α1, even when expressed at twice the levels of that assayed with the wild-type CLD construct (Fig. 7D). Collectively, these data support that the CLD of RanBP2 promotes the biogenesis of selective substrates likely by the modulation of selective, but still unidentified features, of the UPS activity.

**DISCUSSION**

This study shows that the CLD domain of RanBP2 selectively promotes the accumulation of properly folded reporters targeted for degradation by the UPS. This activity of CLD is reflected also by an increase of the steady-state levels of selective RanBP2-interacting substrates, RanGAP and RPRGIP1α1. Remarkably, the CLD affects the levels of substrates that are neither sumoylated nor ubiquitylated, because the molecular species corresponded to the apparent molecular masses of the unconjugated isofoms, and immunoblots with anti-ubiquitin and -SUMO antibodies do not recognize the molecular species whose levels are affected by CLD. This is further supported by the fact that the steady-state level of SUMO-1-RanGAP is not affected by CLD, and the deletion of the ubc9-interacting domain (IR1+2) of RanBP2 does not have a significant impact on the accumulation of the UPS reporter, UbG76V-YFP, when assayed in the presence of CLD and RBD3.

We cannot exclude that a yet unknown sumoylated or unconjugated partner interacts with the purposed SBM of CLD and selectively activates pathways promoting the biogenesis of properly folded proteins and down-regulation of restricted activities of the UPS. Alternatively, sumoylation may antagonize ubiquitylation by rendering the substrates resistant to degradation by the UPS as reported for 1κB and Mdm2 (67, 68). In light of the studies here reported, however, we consider the existence of such scenarios unlikely. First, one would expect such a sumoylated partner to stably and specifically associate with the SBM of CLD and to be detected in the biochemical screening assays for binding partners of CLD as we previously reported (45, 57). Second, although SUMO-1-RanGAP was reported to associate in vitro with SBM of CLD (63), RanGAP has two binding sites for ubc9, and this possibly mediates indirectly the interaction of SUMO-1-RanGAP with RanBP2 (69). In addition, the significance of the intrinsic sumoylation activity of ubc9 is unclear, because it may not reflect, at least in certain functional contexts, its biological activity. Multiple lines of evidence lend support to unrelated roles of SUMO conjugation and ubc9 (E2) in still elusive biological processes. Notably, ubc9 carries functions independent of sumoylation, because a non-enzymatic (catalytic-dead) form of ubc9 is required and sufficient for protein nuclear translocation of Vsx-1, a homeobox homologue of Chx10/Vsx-2 (70). Finally, apparently less than half of ubc-9 interacting proteins are known substrates for sumoylation (71) and as presented in this study, RanGAP is not sumoylated in the retina and the transformed ganglion cell line, RGC-5.

The data presented favor instead a model whereby the CLD promotes the biogenesis of selective substrates possibly by sequestering critical components required for the assembly of the 19S RP of the proteasome and/or shielding the presentation of properly folded, and non- and/or ubiquitylated, substrates for degradation by the UPS (72–74). The synergistic effect of CLD with proteasome inhibitors on the UPS activity also supports the CLD and proteasome inhibitors act on the 26S proteasome by independent mechanisms. In addition to RanBP2, several subunits of the 19S RP are reported to associate with an emerging number of auxiliary partners, and ectopic expression of some of these inhibit the proteolytic activity of the proteasome (18, 19). Hence, the CLD of RanBP2 possibly represents a critical auxiliary factor contributing to the molecular compartmentalization of the modulation of UPS activity on specific substrates, such as a subset of those interacting with RanBP2 (e.g. RanGAP and RPRGIP1α1). Surprisingly, we previously noted that the level of hexokinase type I (HKI) was affected in haplosufficient RanBP2 mice (40), but in the current studies the steady-state level of HKI was not affected upon ectopic expression of CLD. It is possible that the chaperoning activity of the leucine-rich domain of RanBP2 singly modulates the biogenesis of HKI (and Cox11) or that only reduced, but not increased, levels of CLD, affect HKI biogenesis. In this regard, this study is reminiscent to the chaperoning effect of the combined Ran-binding domain 4 and cyclophilin domains (RB4-CY) of RanBP2 on the increased production of functional G-protein coupled receptors, opsins, upon co-expression of these in COS7 cells (43, 44), and to the chaperoning of the homologous *Drosophila* cyclophilin, NinaA, on opsin production and sorting (75–78).

Interestingly, here we observed that the RBD3 counteracted partially the effect of CLD on the UPS. This is important because the four Ran-binding domains of RanBP2 are thought to be functional equivalents of RanBP1 (49). However, reminiscent to the observations reported here and for the RBD4 and CY domains of RanBP2 (43, 44), the yeast RanBP1, Yrb1p, was found to be required for cell cycle-regulated protein degradation and to modulate protein proteolysis (79).

The outcome of this work also raises questions on whether the CLD harbors distinct subdomains and multiple functions. The enhanced binding of S1 and S3 subunits of the 19S RP to the mutant CLD seems contrary to the hypothesis that such increased sequestration of 19S cap subunits should enhance the inhibition of the UPS activity. Hence, it is possible that SBM loss-of-function promotes the dissociation of a putative and modifying factor from CLD that modulates the binding of some of the 19S subunits to CLD. The selective change in the electrophoretic mobility of S5a upon mutation of SBM of CLD suggests such a factor is necessary to mediate the modification of S5a and perhaps to inhibit its recognition by a pool of ubiquitylated substrates. Hence, the post-translational modification of S5a and possibly of other 19S subunits, by CLD may shed light to the mechanisms underlying the CLD-mediated suppression of the UPS.

Finally, the mouse models of RanBP2 recently generated (40) will allow to probe the physiological, functional, and pathological implications of selective loss-of-function of CLD of RanBP2 across biological systems, and the extent of the CLD effects on
the UPS and biogenesis of substrates with critical physiological function. It is likely that a differential impact of CLD exists across experimental biological systems and these will help to validate and further the understanding of concurrent models of mechanisms of modulation and partitioning of the UPS activity, and extend the biological impact of CLD to other protein substrates and diseases processes.

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