Modulation of D-Serine Levels via Ubiquitin-dependent Proteasomal Degradation of Serine Racemase*

Received for publication, March 1, 2006, and in revised form, May 18, 2006. Published, JBC Papers in Press, May 19, 2006, DOI 10.1074/jbc.M601971200

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Mammalian serine racemase is a brain-enriched enzyme that converts L- into D-serine in the nervous system. D-Serine is an endogenous co-agonist at the “glycine site” of N-methyl D-aspartate (NMDA) receptors that is required for the receptor/channel opening. Factors regulating the synthesis of D-serine have implications for the NMDA receptor transmission, but little is known on the signals and events affecting serine racemase levels. We found that serine racemase interacts with the Golgin subfamily A member 3 (Golga3) protein in yeast two-hybrid screening. The interaction was confirmed in vitro with the recombinant proteins in co-transfected HEK293 cells and in vivo by co-immunoprecipitation studies from brain homogenates. Golga3 and serine racemase co-localized at the cytosol, perinuclear Golgi region, and neuronal and glial cell processes in primary cultures. Golga3 significantly increased serine racemase steady-state levels in co-transfected HEK293 cells and primary astrocyte cultures. This observation led us to investigate mechanisms regulating serine racemase levels. We found that serine racemase is degraded through the ubiquitin-proteasomal system in a Golga3-modulated manner. Golga3 decreased the ubiquitylation of serine racemase both in vitro and in vivo and significantly increased the protein half-life in pulse-chase experiments. Our results suggest that the ubiquitin system is a main regulator of serine racemase and D-serine levels. Modulation of serine racemase degradation, such as that promoted by Golga3, provides a new mechanism for regulating brain D-serine levels and NMDA receptor activity.

N-Methyl D-aspartate (NMDA)2 type of glutamate receptors play key roles in excitatory synaptic transmission and are involved in many physiological processes including learning and memory (1). NMDA receptor activity is tightly regulated, as its overactivation contributes to pathologic conditions such as stroke and neurodegenerative diseases (2). An interesting feature of NMDA receptors is the requirement of simultaneous binding of two agonists for channel opening, i.e. the NMDA channel only operates when both a glutamate site and a co-agonist site are occupied (1). It has been shown that binding of glycine to the co-agonist site is an obligatory requirement for NMDA receptor/channel operation (3, 4). Subsequent studies have shown that brain D-serine is an endogenous ligand of the glycine site of NMDA receptors (5–9).

Regulation of NMDA receptor activity by the co-agonist D-serine plays critical roles. Removal of endogenous D-serine decreases NMDA receptor responses (8) and blocks NMDA-dependent migration of immature granule cells in the cerebellum (9). D-Serine is the dominant endogenous co-agonist for NMDA neurotoxicity, as removal of D-serine abolishes NMDA receptor-elicited cell death in hippocampal slices (6). In the vertebrate retina, endogenous D-serine mediates the light-dependent increase in neuronal activity by activating NMDA receptors (10). Furthermore, endogenous D-serine is required for the long term potentiation of the synaptic transmission in the hippocampus, which is thought to be involved in the memory formation (11).

Brain D-serine is synthesized by the serine racemase enzyme, which converts L- into D-serine (12–14). The enzyme occurs in vertebrate retina, endogenous D-serine mediates the light-dependent increase in neuronal activity by converting L- to D-serine, which catalyzes the NMDA receptors (10). In addition to converting L- to D-serine, serine racemase catalyzes the α,β-elimination of water from L-serine to form pyruvate and ammonia (16). This activity was also observed toward D-serine, which seems to limit the achievable D-serine concentration and may play a role in D-serine metabolism as well (17).

Despite the important roles proposed for the endogenous D-serine, little is known about the regulation of its synthesis by serine racemase. Factors altering serine racemase levels will have an impact on NMDA receptor activity, since the D-serine binding site of the receptor is not saturated under normal conditions (1) and serine racemase is key for D-serine homeostasis (5, 13). In the present study we sought to look for proteins that bind and regulate serine racemase using the yeast two-hybrid technique. We discovered that Golga3, also known as GCP170 (18) or golgin-160 (19, 20), strongly binds to serine racemase both in vitro and in vivo. We found that serine racemase is degraded by the ubiquitin-proteasome system and that Golga3 greatly increases serine racemase levels by decreasing its ubiq-
utility and degradation rate. Our data disclose new mechanisms modulating d-serine production, with implications for the regulation of NMDA receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—l-Serine and d-serine were purchased from Bachem. Acetonitrile, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), deoxyribonuclease I, anti-gliial fibrillary acidic protein, anti-microtubule-associated protein 2 antibodies, and imidazole, were obtained from Sigma. Monoclonal anti-hemagglutinin (HA), anti-HA matrix, and anti-Myc matrix were obtained from Covance. Monoclonal anti-Myc was obtained from Calbiochem. Polyclonal anti-Myc was obtained from Santa Cruz Biotechnology. Monoclonal anti-GM130 was from BD Biosciences. Basal Medium Eagle, minimum essential medium, fetal bovine serum, glutamine, penicillin-streptomycin, trypsin, and soybean trypsin inhibitor were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). B27 supplement was obtained from Invitrogen. Other reagents were of analytical grade.

**Yeast Two-hybrid Screening**—The yeast two-hybrid experiments were carried out as previously described (21) using full-length mouse serine racemase cloned into ppC97 as bait and screening both a rat hippocampal and a human fetal brain library in ppC86 (provided by Prof. P. Worley, Johns Hopkins University). Positive interactors were identified by their ability to grow on selective plates (Trp− Leu− His−) and activate the transcription of the reporter gene, β-galactosidase.

**Cell Culture and Transfection**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. For transfection, cells were plated into 6-well tissue culture plates (Nunc) at 70–90% confluence. On the next day cells were transfected with mouse full-length serine racemase in pRK5 containing HA in the N terminus (13), Golga3-Myc (golgin-160) in pcDNA 3.1 (provided by Prof. C. E. Machamer, Johns Hopkins University). Positive interactors were identified by their ability to grow on selective plates (Trp− Leu− His−) and activate the transcription of the reporter gene, β-galactosidase.

**In Vitro Binding Assays**—Extracts of transfected HEK293 cells were incubated with 5 μg/ml GST or GST-serine racemase (22) bound to glutathione-Sepharose beads for 1 h at 4°C in buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1% Triton X-100, and protease inhibitors (Complete, Roche Diagnostics). The beads were washed 5 times with the same buffer supplemented with 0.3 M NaCl, and bound material was resolved by SDS-PAGE and detected by Western blot analysis.

**Co-immunoprecipitation Assays**—Transfected HEK293 cells were lysed by sonication in buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and protease inhibitors (Complete, Roche Diagnostics). Then 0.1% Triton X-100 was added, and the cell extract was cleared by centrifugation at 18,000 × g for 10 min. The supernatant was incubated for 4 h at 4°C with Myc affinity matrix (Covance), and the immunoprecipititates were washed 6 times with lysis buffer supplemented with 0.3 M NaCl. The blots were probed with monoclonal anti-HA (1:2000) of polyclonal anti-Myc (1:2000). For co-immunoprecipitation of endogenous proteins, rat brains were homogenized using a glass homogenizer with 5 volumes of 20 mM Tris-HCl (pH 7.4), 0.3 M NaCl, 0.5% Triton X-100, and protease inhibitors (Complete, Roche Diagnostics). The homogenate was cleared by centrifugation (40,000 × g) for 40 min. Ten microgram of purified antibodies to serine racemase and glucosamine-6-phosphate deaminase (23) were coupled to protein G beads using dimethylpyrrolidinomethylate as previously described (24) and incubated for 12 h with rat brain homogenate (1.5 mg/ml). After washing 7 times with lysis buffer containing 0.5% Triton X-100 and 0.5 M NaCl, the immunoprecipitates were revealed with anti-GCP170 (Golga3) polyclonal antibody generated by Misumi et al. (18).

**Brain Subcellular Fractionation**—Subcellular fractionation was carried out as described by Huttner et al. (25). Brains from adult Sprague-Dawley rats were homogenized in 5 volumes of buffer containing 5 mM Heps-NaOH (pH 7.3), 0.32 M sucrose, 0.4 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of aprotinin, leupeptin, chymostatin, and pepstatin. The homogenate was centrifuged at 800 × g for 15 min to give P1 (crude nuclear pellet) and S1 (supernatant). S1 was centrifuged at 9200 × g for 15 min to give P2 (crude synaptosomal/mitochondrial pellet) and S2. P3 (microsomal pellet) and S3 (cytosolic fraction) were obtained by centrifuging S2 at 120,000 × g for 1 h. P3 washed (washed microsomal pellet) was obtained by washing the P3 membranes in 50 ml of buffer containing 20 mM Tris-HCl (pH 7.4) and 0.3 M NaCl to remove loosely bound proteins. Samples were analyzed by SDS-PAGE and Western blot, and probed with antibodies anti-Golga3 (1:1000 – 1:2000), anti-SR serum (1:5000), and anti GM130 (1:2500).

**Enzyme Markers**—Lactate dehydrogenase activity was determined by monitoring the conversion of pyruvate to lactate coupled to oxidation of NADH (26). Fumarase activity was determined by monitoring the conversion of malate into fumarate by a photometric assay (26).

**Primary Cell Cultures**—Animals were killed by quick decapitation with the approval of the Committee for the Supervision of Animal Experiments (Technion-Israel Institute of Technology). Neuronal cultures from cerebral cortex were prepared from E16-E18 Sprague-Dawley embryos as previously described (27). Nearly pure glial cultures were prepared from post-natal day 3 rat pups using Basal Medium Eagle containing 10% fetal bovine serum. The cells were vigorously washed with Hank’s-buffered saline solution 4–5 times every 2 days to dislodge neuronal cells and microglia. Glial cultures were used 10–14 days after plating.

**Assay of d-Serine Synthesis**—HEK293-transfected cells were incubated in 6-well plates with 1 ml of Dulbecco’s modified Eagle’s medium. d-Serine was measured in the culture medium by high performance liquid chromatography as previously described (16).

**Immunocytochemistry Assays**—Primary neural culture cells were fixed with 4% paraformaldehyde for 20 min. The cells were blocked and permeabilized with 8% normal goat serum, 0.1% Triton X-100 in Tris-buffered saline (50 mM Tris-HCl (pH 7.4) and 150 mM NaCl). Primary antibody incubation was carried out for 12–16 h at 4°C with the polyclonal antibody to serine racemase (Ab2, 0.3 μg/ml) previously characterized (15), monoclonal anti-GM130 (1:200, BD Biosciences), and serum anti-GCP170 (Golga3) (1:150). Immunolabeling was detected
by using Cy2- and Cy3-labeled secondary antibodies (Jackson Immunoresearch Laboratories). For double-labeling with polyclonal antibodies (serine racemase and Golga3), serine racemase was first labeled using Cy3-conjugated anti-Fab fragment anti-rabbit IgG that blocks all the primary antibody sites. Then rabbit anti-GCP170 (Golga3) was added followed by Cy-2-conjugated anti-rabbit IgG. Co-localization was analyzed by confocal laser microscopy.

**Lentivirus Production**—N-terminal HA-tagged Golga3 (455–888 fragment) was subcloned into pTK208 lentiviral vector (a gift from Dr. T. Kafri, Univ. of North Carolina, Chapel Hill) containing cytomegalovirus promoter. The virus was produced by calcium-mediated co-transfection of the lentiviral vector (5 μg), packing vector pCMV-DR8.74 (3 μg), and vesicular stomatitis virus glycoprotein envelope pMD2G (2 μg) (a gift from Prof. D. Trono, University of Geneva) in HEK293 cells grown in 10-mm culture dishes (29). Control virus consisted of GFP under the control of cytomegalovirus promoter. Viral stocks were produced by concentrating the viral particles present in the culture medium by centrifugation at 120,000 × g for 2 h. The pellet was washed once by centrifugation with fresh culture medium and suspended in a small volume of basal medium Eagle culture medium. The viruses were stored at −70°C until use. Virus titer was determined by infecting HEK293 cells and monitoring the immunofluorescence for GFP or immunocytochemistry for HA-Golga3 (455–880) with anti-HA antibody. To infect primary astrocyte cultures, viral stocks were added to cultured cells grown in 24-well plates, and the medium was replaced after 48 h. The experiments were carried out 5 days after infection under conditions that virtually all cells had been infected.

**In Vitro Ubiquitylation**—Mouse full-length serine racemase was translated in vitro by the use of Wheat germ extract in vitro translation system (Promega) and [H3]methionine (Amersham Biosciences). For in vitro ubiquitylation, 1 μl of the translated protein was incubated with 40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 5 mM MgCl₂, 1 μM ubiquitin aldehyde, 0.6 mg/ml ubiquitin (Sigma), 2 mM ATPγS (Sigma), and 4 mg/ml transfected HEK293 extract for 60 min at 37°C. Controls were carried out by omitting ATPγS and removing endogenous ATP with 20 mM deoxyglucose and 240 units/ml of hexokinase (Sigma). The ubiquitin-protein conjugates were resolved by SDS-PAGE followed by phosphorimaging analysis. For producing HEK293 cell extracts, the cells were transfected with Golga3-Myc in pC DNA 3.1 using Polyfect reagent (Qiagen). Forty-eight hours after transfection, the cells were harvested and lysed by 2 freeze/thaw cycles in liquid N₂ in medium containing 20 mM Tris-HCl (pH 7.4), 0.5 M MgCl₂, 1 mM dithiothreitol, 5 mM KCl, 50 mM HEPES, 5 mM dithiothreitol, deoxyribonuclease I (10 units/ml), 0.4 mM phenylmethylsulfonyl fluoride, 30 μM MG132, and 1 μg/ml leupeptin, pepstatin, aprotinin, and chymostatin. The suspension was cleared by centrifugation at 16,000 × g for 40 min and diluted 10-fold into buffer containing 2% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, and protease inhibitor mixture. After additional centrifugation to remove any insoluble material, immunoprecipitation was carried out with anti-HA affinity-matrix and washed 6 times with radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5)). Then the ubiquitin-serine racemase conjugates were revealed with polyclonal anti-FLAG (Sigma).

**Pulse-chase Experiments**—HEK293 cells were transfected with HA-SR and either Golga3-Myc or GFP at a SR/Golga3 cDNA ratios of 1:5 to 1:9. Forty-eight hours after transfection, the medium was replaced by Dulbecco’s modified Eagle’s medium lacking methionine/cysteine (Sigma) for 1 h. Then the cells were pulsed with methionine/cysteine-free medium containing 100 μCi of [³⁵S]methionine/cysteine (PerkinElmer Life Sciences) for 70 min and subsequently chased in complete Dulbecco’s modified Eagle’s medium. At the specified times, the cells were harvested and lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1% Triton X-100, 30 μM MG132, and protease inhibitors (Complete, Roche Diagnostics). Immunoprecipitation of HA-serine racemase was carried out with anti-HA affinity matrix (Covance), and the immunoprecipitates were washed six times with radioimmune precipitation assay buffer. The samples were resolved on SDS, 10% PAGE gels, transferred to nitrocellulose membranes, and quantified by phosphorimaging analysis. The amount of immunoprecipitated HA-serine racemase was checked by Western blot of the membrane followed by densitometry of the chemiluminescent signal.

**RESULTS**

**SR Interacts with Golga3**—We identified Golga3 protein as a novel interactor of serine racemase in yeast two-hybrid screenings (Fig. 1A). Two independent clones of Golga3 were identified in screenings of different libraries and encompass the amino acid regions 455–880 and 180–665 (Fig. 1B). Golga3 associates with the cytosolic surface of the Golgi apparatus, where it may be involved in vesicular trafficking (31, 32). Analysis of the primary amino acid sequence indicates that the interacting region of Golga3 (amino acids 455–665) is predicted to adopt a coiled-coil amino acid sequence indicates that the interacting region of serine racemase using yeast two-hybrid (Fig. 1A). We further mapped the interacting region of serine racemase using yeast two-hybrid (Fig. 1C). As a positive control, we observed that co-transformation of fos and jun strongly activated β-galactosidase expression (Fig. 1A). We further mapped the interacting region of serine racemase using yeast two-hybrid (Fig. 1C). As a positive control, we observed that co-transformation of fos and jun strongly activated β-galactosidase expression (Fig. 1A). We further mapped the interacting region of serine racemase using yeast two-hybrid (Fig. 1C). As a positive control, we observed that co-transformation of fos and jun strongly activated β-galactosidase expression (Fig. 1A).
Serine Racemase Is Degraded through the Proteasome

| Bait   | Prey          |
|--------|--------------|
| SR     | Golga3 (aa455-880) |
| SR     | Golga3 (aa180-665) |
| FOS    | JUN          |
| PPC97  | Golga3 (aa455-880) |
| PPC97  | Golga3 (aa180-665) |
| PPC86  | Rac          |

C. Constructs:

- Serine Racemase (1-339)
- Serine Racemase (1-335)
- Serine Racemase (1-134)
- Serine Racemase (1-66)

D.

| Input (5%) | GST | GST-SR |
|------------|-----|--------|
| Golga3-Myc |     |        |

E.

- HA-SR (+ + +)
- Myc-LacZ (+ - -)
- Golga3-Myc (- + -)
- Golga3-Myc (aa455-880) (- - +)

F.

| Input (2%) | IP GNPDA | IP SR |
|------------|----------|------|
| kDa        | 150-     | 37-  |
| Golga3     |          |      |
| SR         |          |      |
Serine Racemase Is Degraded through the Proteasome

To confirm the interaction, we carried out GST binding assays. Full-length Golga3 construct containing a C-terminal Myc tag (20) was transfected into HEK293 cells, and the cell extract was incubated with beads containing GST-serine racemase (GST-SR). The interaction of Golga3 with serine racemase was confirmed by the appearance of GST-SR bound to GST-SR but not to GST alone (Fig. 1D).

The interaction of Golga3 with serine racemase was also confirmed in intact cells by co-transfecting HA-serine racemase (HA-SR) with Golga3-Myc (full-length and 455–880 fragment) or Myc-LacZ control cDNA in HEK293 cells. The Myc-tagged proteins were immunoprecipitated with anti-Myc, and the blot was probed with anti-HA. Serine racemase robustly co-immunoprecipitated with Golga3 constructs but not with LacZ (Fig. 1E).

To verify that the proteins interact in vivo, we carried out co-immunoprecipitation experiments from rat brain extracts. Beads coupled with purified antibody to serine racemase specifically co-immunoprecipitated Golga3 (Fig. 1F, upper panel). To ensure specific binding, the immunoprecipitates were washed with high salt buffer containing 0.5% Triton X-100 and 0.5 M NaCl. Furthermore, beads coupled with an antibody against the unrelated protein glucosamine-6-phosphate deaminase did not co-immunoprecipitate Golga3 (Fig. 1F, upper panel). This suggests that Golga3 and serine racemase specifically interact in vivo.

Subcellular Distribution of SR and Golga3—In case the interaction is physiologically relevant, serine racemase and Golga3 should display overlapping localizations. We first examined the subcellular localizations of endogenous Golga3 and serine racemase in brain extracts. In the brain we detected enrichment of Golga3 in high speed membrane fraction (P3) (Fig. 2A). A significant fraction of Golga3 remained bound to the membrane even after washing with buffer containing high salt (P3 washed), indicating a strong interaction with membranes (Fig. 2A). It is noteworthy that significant levels of Golga3 were also found in the...
Serine Racemase Is Degraded through the Proteasome

Transf. HEK293

Neu

Neu

Ast

Ast
cytosolic fraction of brain (Fig. 2A). Some bands of smaller molecular weight are also seen in the cytosolic fraction. This may be related to the previously described partial cleavage of Golga3 that releases it from the Golgi membrane (20, 33). Serine racemase was also observed in both soluble and membrane fractions of the brain (Fig. 2B). Although most serine racemase is cytosolic, a portion of serine racemase protein is present in the microsomal fraction (P3) of brain (Fig. 2B). Association of serine racemase to the membrane fraction is strong, since washing the membranes with high salt solution did not remove the membrane-bound enzyme (Fig. 2B, compare P3 with washed P3).

We found that the cis-Golgi matrix protein GM130 is highly enriched in P3 and washed P3 fraction, whereas only trace levels were found in the cytosolic fraction (Fig. 2C). This indicates that Golgi membrane fragments are enriched in P3 fraction and that our cytosolic fraction is mostly devoid of membranes. In addition, we monitored the activity of the enzymes lactate dehydrogenase and fumarase, which are specific markers for cytosol and mitochondria matrix, respectively. We found that lactate dehydrogenase activity is clearly enriched in the cytosol and virtually absent from the P3 fractions, indicating that our microsomal preparation is devoid of cytosolic contaminants (Fig. 2C). The fumarase activity was enriched in P2, which corresponds to a fraction enriched in mitochondria and synaptosomes, whereas only traces were detected in P3 (Fig. 2C). These findings are consistent with the notion that a part of the racemase molecules in cells may be specifically associated with microsomal membrane containing Golga3.

Co-localization of SR and Golga3—In co-transfected HEK293 cells, serine racemase and Golga3 exhibited co-localization both at the cytosol and perinuclear region (Fig. 3, A–C). Serine racemase was distributed throughout the cytosol (Fig. 3A). In some cells, a cytosolic distribution of Golga3 was also apparent (Fig. 3B, arrow), whereas in others Golga3 was concentrated in the perinuclear region with lighter staining of the cytosol (Fig. 3B). This pattern of Golga3 distribution in transfected cells was also observed in the absence of serine racemase co-transfection (data not shown).

To confirm that parts of the endogenous proteins co-localize, we analyzed the localizations of Golga3 and serine racemase in primary neuronal cultures. Consistent with our recent description of serine racemase in primary neuronal cultures using a new antibody to serine racemase (15), we found the enzyme to be present in the cytosol as well as in neuronal processes (Fig. 3D). Likewise, Golga3 was detected throughout the cytosol and neuronal processes and co-localized to a large extent with serine racemase (Fig. 3, E and F). The identity of the cells as neurons was routinely checked with an antibody to microtubule-associated protein 2 (data not shown). To further evaluate possible co-localization of serine racemase and Golga3 at the Golgi membrane, we carried out double-labeling with the specific Golgi marker GM130. This revealed that a significant fraction of endogenous serine racemase and Golga3 co-localized at the Golgi apparatus in primary neuronal cultures (Fig. 3, G–L). A similar pattern was observed in primary astrocyte cultures (Fig. 3, M–R). Astrocyte cultures were positive for the astrocytic marker glial fibrillary acidic protein (data not shown). Together with the immunoprecipitation data (Fig. 1), the results indicate that serine racemase and Golga3 interact in vivo and display partially overlapping localizations at the cytosol and at the Golgi apparatus.

Golga3 Increases Steady-state SR Levels—To investigate the role of serine racemase-Golga3 interaction, we first carried out co-transfection studies in HEK293 cells. Interestingly, we observed that Golga3-Myc promoted a dose-dependent increase in serine racemase steady-state protein levels in comparison to cells co-transfected with GFP (Fig. 4, A and B). As additional control, we investigated the effect of the Golga3 N-terminal fragment (aa 1–380) that does not interact with serine racemase in yeast two-hybrid assays (data not shown). As shown in Fig. 4C, this non-interacting construct did not increase the levels of serine racemase (lanes 3 and 4) as compared with the full-length Golga3-Myc (lanes 1 and 2). The striking increase in steady-state serine racemase levels promoted by Golga3-Myc was associated to a robust increase in D-serine synthesis, which accumulated in the culture media (Fig. 4D). This indicates that Golga3 promotes the accumulation of a functional serine racemase enzyme. Co-transfection of the Golga3 fragment (aa 455–880) isolated in the yeast two-hybrid screening also promoted an increase in both serine racemase levels and D-serine synthesis in HEK293 cells (data not shown).

To assure that Golga3 modifies endogenous levels of serine racemase, we produced recombinant lentivirus carrying the cDNA of the Golga3 fragment (aa 455–880) that interacts with serine racemase and investigated its effects on serine racemase expression in primary astrocyte cultures. Injection of the cultures with Golga3 fragment doubled the steady-state levels of endogenous serine racemase levels when compared with lentivirus containing GFP (Fig. 4 E and F). Actin levels were unchanged, indicating the specificity of the effect (Fig. 4E).

Proteasomal Degradation of SR—To investigate the mechanisms regulating serine racemase levels and the role of Golga3, we first tested whether serine racemase is degraded by the ubiquitin-proteasome system, which mediates selective degradation of many short-lived proteins (34). The addition of the proteasome inhibitor MG132 more than doubled the steady-state levels of endogenous serine racemase in primary astrocyte cul-

**FIGURE 3. Co-localization of serine racemase, Golga3, and GM130 in transfected cells and primary cultures.** A–C, co-localization of serine racemase (A) and Golga3 (B) in transfected HEK293 cells analyzed by confocal laser microscopy. Significant co-localization was observed in the perinuclear region. In a fraction of the cells Golga3 was observed in the cytosol (panel B, arrow), where it also co-localized with serine racemase. D–F, co-localization of endogenous serine racemase (D) and Golga3 (E) at the neuronal somata and processes of cortical primary neuronal cells (Neu). G–I, co-localization of endogenous serine racemase (G) and GM130 (H) in the perinuclear region of neurons. J–L, co-localization of endogenous Golga3 (U) and GM130 (K) in the perinuclear region of neurons. M–O, co-localization of endogenous serine racemase (M) and Golga3 (N) at the cytosol and glial processes of cortical primary astrocyte cells (Ast). P–R, co-localization of endogenous Golga3 (P) and GM130 (Q) in the perinuclear region of astrocytes. The bar represents 10 μm. Ast, primary astrocyte culture; Neu, primary neuronal culture; Transf., transfected HEK293 cells.
Serine Racemase Is Degraded through the Proteasome

**A**

|          | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
|----------|-----|-----|-----|-----|-----|
| HA-SR    |     |     |     |     |     |
| GFP      | 0.9 | 0.8 | 0.6 | 0.4 | -   |
| Golga3-Myc | -   | 0.1 | 0.3 | 0.5 | 0.9 |

**B**

Golga3-Myc

| O.D. ( Arbitrary units) |
|-------------------------|
| 0.1                     |
| 0.3                     |
| 0.5                     |
| 0.9                     |

**C**

|          | 0.1 | 0.1 | 0.1 | 0.1 |
|----------|-----|-----|-----|-----|
| HA-SR    |     |     |     |     |
| Golga3-Myc | 0.9 | 0.9 | -   | -   |
| Golga3-Myc | (1-380) | -   | 0.9 | 0.9 |

**D**

Golga3-Myc

| nmol D-serine/mg prot. |
|------------------------|
| 450                    |

**E**

GFP Virus

**F**

HA-Golga3 (555-880)

| O.D. Rac/Actin (AU) |
|---------------------|
| 0.6                 |

| HA-Golga3 (555-880) |
|---------------------|
| 0.6                 |

investigated the effect of HEK293 cell extracts from cells overexpressing Golga3 and monitored serine racemase in vitro ubiquitylation (Fig. 6A). Inspection of the ubiquitylated conjugates revealed that Golga3 decreased the formation of the higher molecular weight conjugates of serine racemase, located at the top of the gel (Fig. 6A, region a). The higher molecular weight conjugates are formed by large serine racemase-polyubiquitin conjugates, which are more efficiently degraded by the proteasome when compared with lower molecular weight conjugates (35). Golga3 did not affect the

cases, whereas actin levels were unchanged (Fig. 5, A and B). Another proteasomal inhibitor, lactacystin (10 μM), also significantly increased serine racemase levels (data not shown). Thus, serine racemase seems to be degraded through the proteasome. In transfected HEK293 cells, treatment with MG132 also increased serine racemase levels when co-transfected with the control protein LacZ (Fig. 5C). However, MG132 did not promote additional serine racemase accumulation beyond that observed by co-transfection with Golga3 (Fig. 5, C and D). Thus, we wondered whether Golga3 could increase serine racemase levels by slowing down serine racemase degradation through the ubiquitin proteasome system. MG132 is known to cause robust accumulation of polyubiquitin-protein conjugates due to proteasomal inhibition (34). These conjugates, however, were not observed in the Western blot of Fig. 5C, possibly because the polyubiquitin chains might conceal the epitopes recognized by the antibody to serine racemase. To directly monitor the effects of Golga3 in the ubiquitylation and degradation rate of serine racemase, we carried out ubiquitylation studies (Fig. 6) and measurement of serine racemase half-life (Fig. 7).

Golga3 Decreases SR Ubiquitylation—We first investigated if serine racemase is a substrate for the ubiquitin system by employing an in vitro ubiquitylation assay. Serine racemase was translated in vitro and incubated with ubiquitin and HEK293 cell extracts to provide the other components of the ubiquitin system. We observed formation of high molecular weight species compatible with polyubiquitylation of serine racemase (Fig. 6A). As expected, the process was ATP-dependent, as removal of ubiquitin proteasome. We observed formation of high molecular weight species compatible with polyubiquitylation of serine racemase (Fig. 6A). As expected, the process was ATP-dependent, as removal of ATP decreased the ubiquitylation to a large extent. To evaluate the role of Golga3, we

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Serine racemase, Golga3, or GFP control. Serine racemase was immunoprecipitated under denaturing conditions to avoid co-immunoprecipitation of other ubiquitylated proteins. Polyubiquitination was detected with anti-FLAG antibody. Under these conditions we observed that Golga3 also decreased the in vivo ubiquitination of serine racemase by about 50% (Fig. 6C).

Golga3 Increases SR Protein Half-life—It is conceivable that the decrease in serine racemase ubiquitination by Golga3 will lead to an increase in serine racemase half-life by decreasing the ubiquitin-dependent proteasomal degradation of serine racemase. To verify this possibility, we monitored the turnover of serine racemase by chase experiments after labeling the cells with [35S]methionine (Fig. 7). Serine racemase was detected by immunoprecipitation followed by phosphorimaging analysis (Fig. 7A). We found that serine racemase has a relatively short half-life of about 4.5 h (Fig. 7B). Co-transfection of Golga3 significantly decreased the rate of serine racemase degradation by promoting an almost 3-fold increase in its half-life (Fig. 7B). This indicates that the effect of Golga3 is mediated by increasing serine racemase protein stability and not through effects on gene transcription.

To evaluate the specificity of Golga3 action, we investigated if it affects the degradation of another substrate of the ubiquitin system. For this, we employed GFPu, which is an unstable reporter (t1/2 = 30 min) consisting of a short degron fused to the C terminus of green fluorescent protein that is degraded by the proteasome (36). Global changes in the ubiquitin-proteasomal system will likely affect the levels of GFPu. Golga3 had no effect on steady-state levels of GFPu, indicating that Golga3 does not promote a general inhibition of the ubiquitin-proteasomal system (Fig. 7C).

**DISCUSSION**

In the present work we identified Golga3 as a new serine racemase binding partner. The interaction we described reveals new mechanisms regulating the levels of serine racemase and the neuromodulator D-serine. We discovered that serine racemase is ubiquitylated both in vitro and in vivo, which leads to its degradation by the ubiquitin-proteasome system. Golga3 modulates the ubiquitylation of serine racemase, which significantly increases its half-life and steady-state levels.

Golga3 was first identified as an autoantigen in an autoimmune disease (19). It contains several functional domains, including an N-terminal non-coiled-coil domain followed by an extensive coiled-coil region analogous to the myosin family (18, 33). The N-terminal domain contains both a Golgi targeting signal and a polyubiquitin-binding site (33). The N-terminal region of Golga3 (golgin-160) is cleaved by caspases during apoptosis, which may be important for cessation of membrane trafficking, Golgi disassembly, and possibly initiation of apoptosis (20, 37). Golga3 cleavage is enhanced upon phosphorylation by the mixed lineage kinase 3, a potent activator of the c-Jun NH2-terminal kinase pathway (38). Cells expressing a caspase-resistant mutant of Golga3 are resistant to apoptosis induced by ligation of death receptors and by drugs that induce endoplasmic reticulum stress, indicating that Golga3 plays an important role in transduction of some apoptotic signals (37). Recent studies also indicate a role of Golga3 in membrane traffick-
ing. When expressed in *Xenopus* oocytes, Golga3 facilitates the transport of the renal inward rectifier potassium channel to the cell surface (39). Golga3 was shown to interact with PIST, a protein involved in vesicular trafficking (32). GCP170, a 32-amino acid longer version of Golga3, binds to GCP16, an acylated Golgi-associated protein thought to be involved in trafficking of cargo to the plasma membrane (31). Despite the above studies, the role of Golga3 is still unknown, as no specific function has been ascribed to this widely expressed protein.

A role of Golga3 in processes unrelated to classic Golgi function has not been previously investigated, although part of Golga3 was observed in the cytosolic fraction of HeLa cells (18). We now describe an interaction between Golga3 and serine racemase both *in vitro* as well as *in vivo* by co-immunoprecipitation studies (Fig. 1). The interaction is mediated by the coiled-coil domain of Golga3, as both isolated clones lack the N-terminal non-coiled region (Fig. 1). We observed Golga3 in the cytosolic fraction of brain homogenates (Fig. 2) and also in the cytosol and processes of neuronal and glial cells (Fig. 3). This suggests an additional function for Golga3 unrelated to its presence on the cytosolic surface of the Golgi apparatus.

An intriguing observation is the presence of serine racemase strongly bound to the membrane fraction, which was resistant to washing with high salt concentration (Fig. 2). The presence of serine racemase in the perinuclear region corresponding to the Golgi apparatus was confirmed by co-localization studies with a Golgi marker (Fig. 3). Thus, it is possible that Golga3 mediates serine racemase binding to the Golgi membrane. The role of Golgi-associated serine racemase is unclear, since no intracellular target for d-serine has been identified so far. Nevertheless, membrane-bound serine racemase displays racemization activity, indicating that it may constitute a functional reservoir of the enzyme to be exported to other places in the cell. In view of the fact that d-serine has been observed in vesicle-like structures in some types of cells (40, 41), it is tempting to speculate that membrane-bound serine racemase could play a role in the cellular compartmentation or release of d-serine.

Our present work reveals a role for Golga3 in modulating serine racemase levels (Fig. 4, A–D). Golga3 also increased the steady-state levels of serine racemase in primary astrocyte cultures, indicating that it affects the endogenous serine racemase levels (Fig. 4E). We found that the proteasomal activity regulates serine racemase levels (Fig. 5), and Golga3 promoted a decrease in serine racemase ubiquitylation both *in vitro* and *in vivo* (Fig. 6). The data suggest that a decrease in polyubiquitylation of serine racemase accounts for the increase in the steady-state levels (Fig. 4) and half-life of serine racemase (Fig. 7) promoted by Golga3.

It has been shown that the ubiquitin system regulates trafficking of cargo from Golgi, and some E3 ubiquitin ligases are located in this organelle (42–44). However, Golga3 has not been previously implicated in the modulation of protein ubiquitylation. One possible explanation for Golga3 action is its interference with the binding of a still unidentified E3 ubiquitin ligase to serine racemase, a class of proteins that promote the transfer of ubiquitin to proteins and ubiquitin chains (34). Despite the fact that Golga3 did not affect GFP*u* levels (Fig. 7C), it is possible that Golga3 affects the half-life of additional proteins by a mechanism similar to that observed for serine racemase. The metabolism of d-serine in the forebrain areas is slow due to the scarcity of the metabolic enzyme d-amino acid oxidase enzyme in this region (7). The limited metabolism of d-serine may account for the long half-life of d-serine in the brain, which was calculated to be about 12 h (45). On the other hand, we found that serine racemase displays a relatively short half-life of about 4.5 h (Fig. 7), which is compatible with the existence of an efficient degradation/regulatory system. In this framework, regulation of serine racemase levels by the ubiquitin system and through its interaction with Golga3 might play a key role in regulating d-serine synthesis.

The co-agonist site of the NMDA receptors that is occupied by d-serine is an important regulator of the receptor/channel function (1). As an endogenous co-agonist, d-serine plays...
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important roles in regulating NMDA receptor responses and neurotoxicity (6, 8–11, 46). On the other hand, the ubiquitin system has been implicated in protein turnover at synaptic sites, but the mechanisms regulating synapctic proteins by degradation only started to be unveiled (47). Synaptic scaffold proteins such as Shank, AKA7P9/150, and guanylate kinase-associated protein (GKAP) are ubiquitylated in an activity-dependent manner and may regulate the targeting of NMDA receptors at the synapse (48). Although the NMDA receptor by itself can be ubiquitylated (28), its half-life is quite long (about 20 h (37)). Our data raise the possibility that the ubiquitylation of serine racemase and its modulation by Golga3 are likely to play a role in NMDA receptor activity and neurotoxicity as well.

In summary, our study discloses new mechanisms regulating the synthesis of the neuropomodulator D-serine, with implications for the regulation of NMDA activity and neurotoxicity. Identification of the E3 ligase responsible for serine racemase ubiquitylation in future studies will allow a more complete understanding of the mechanism regulating brain D-serine levels.

Acknowledgments—We thank to Prof. Avram Hershko (Technion-Israel Institute of Technology) for helpful discussions and for providing ATP-β-S and ubiquitin aldehyde and Drs. Ofer Shenker and Edith Suss-Toby for expert technical assistance with confocal imaging. We are also indebted to Dr. Michael J. Schell (Uniformed Services University of the Health Sciences) for helpful discussions and SR-GFP constructs and to Dr. Carolyn Machamer (Johns Hopkins University) for golgin-160/Golga3 cDNA.

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